

**IMPROVED ELECTROPHYSIOLOGICAL ASSAYS USING OOCYTES THAT  
EXPRESS HUMAN ENaC AND THE USE OF PHENAMIL TO IMPROVE  
THE EFFECT OF ENaC ENHANCERS IN ASSAYS USING MEMBRANE  
POTENTIAL REPORTING DYES**

**5 Cross Reference to Related Applications**

[0001] This application claims priority from U.S. Provisional Application Serial No. 60/485,745 filed July 10, 2003, US Provisional Application Serial No. 60/287,413, filed May 1, 2001, and to US Utility Application Serial No. 10/133,573 filed April 29, 2002, all of which are incorporated herein by reference in their entireties.

[0002] The present invention involves the discovery that the efficacy of cell-based assays that screen for compounds that modulate ENaC function, preferably ENaC enhancers, is improved by the further use of a compound that at least partially inhibits ENaC function preferably an amiloride derivative such as phenamil. The present invention further relates to improved electrophysiological assays that identify human ENaC modulators using oocytes, preferably frog oocytes, that express a functional human ENaC sodium channel.

**Field of the Invention**

[0003] The present invention in part relates to novel cell based assays that use recombinant host cells that express amiloride-sensitive sodium channels to profile, screen for, and identify taste modulating compounds. More specifically, the invention relates to assays that utilize test cells that express a functional human epithelial sodium channel (hENaC), preferably amphibian oocytes or mammalian cells, and the use of these test cells in cells in high throughout or moderate throughput cell-based assays, preferably

electrophysiological assays, to identify compounds that enhance or block hENaC function.

### **Background of the Invention**

[0004] An amiloride-sensitive epithelial sodium channel (ENaC) mediates 5 sodium influx across the apical membrane of taste buds cells in the tongue (Heck, et al, Science (1984) 223: 403-405). ENaC, a member of the ENaC/degenerin superfamily of ion channels involved in sodium transport, is composed of three partially homologous  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits expressed at both the RNA and protein level in fungiform, foliate, and circumvallate papilla 10 as well as the lingual epithelium in taste tissue (Li, et al, Proc. Natl. Acad. Sci. (1994) 91: 1814-1818; Kretz, et al, J. Histochem. Cytochem. (1999) 47(1): 51-64; Lin, et al, J. Comp. Neurol. (1999) 405: 406-420; Xiao-Jiang, et al, Mol. Pharmacol. (1995) 47: 1133-1140).

[0005] Complementary DNAs (cDNAs) encoding amiloride-sensitive 15 epithelial sodium channel (ENaC) channel subunits have been isolated from kidney cells and expressed in a mammalian cell line. The channel expressed in this system has been shown to have similar properties to the distal renal sodium channel, *i.e.*, high sodium selectivity, low conductance, and amiloride sensitivity. One form of the naturally occurring ENaC channel is comprised of 20 three subunits of similar structure: alpha (OMIM Entry 600228), beta (OMIM Entry 600760), and gamma (OMIM Entry 600761). Each of the subunits is predicted to contain 2 transmembrane spanning domains, intracellular amino- and carboxy-termini, and a cysteine-rich extracellular domain. The three subunits share 32 to 37% identity in amino acid sequence. Alternatively

spliced forms of alpha-ENaC have also been identified, indicating heterogeneity of alpha subunits of amiloride-sensitive sodium channels that may account for the multiple species of proteins observed during purification of the channel (See US Pat No. 5,693,756, which is herein incorporated by 5 reference).

[0006] An inhibitor of ENaC sodium channel function, amiloride, is known to attenuate gustatory responses to sodium chloride in numerous non-mammalian as well as mammalian species, including humans (Halpern, Neuroscience and Behavior Reviews (1998) 23: 5-47 and all references cited 10 within; Liu, et al, Neuron (2003) 39: 133-146; Zhao, et al, Cell (2003) 115: 255-266). In humans, amiloride reportedly reduces the intensity of sodium chloride by 15-20% when used at concentrations that specifically inhibit ENaC function (Halpern, Neurosciences and Behavior Reviews (1998) 23:5-47 and all references cited within; Feldman, et al, J. Neurophysiol. (2003) 90(3): 15 2060-2064). Therefore, compounds that increase the transport of sodium ions through ENaC channels may function as general salt taste enhancers and augment human salt taste perception as suggested in our previous patent application (PCT WO 02/087306 A2). Further, based on published electrophysiological data and the discovery that a human ENaC is expressed 20 in taste bud cells, a model of salty taste transduction mediated by ENaC has been constructed. As such, the use of ENaC in the identification of substances which stimulate or block salty taste perception has been suggested (See US Pat. No. 5,693,756, *supra* and PCT WO 02/087306 A2).

[0007] Cell-based functional expression systems commonly used for the physiological characterization of ENaC are *Xenopus laevis* oocytes and cultured mammalian cell lines. The oocyte system has advantages over mammalian cells in that it allows the direct injection of multiple mRNAs, 5 provides high levels of protein expression, and can accommodate the deleterious effects inherent in the over expression of ENaC. However, the drawbacks of this system are that electrophysiological recording in *Xenopus* oocytes is not amenable to screening large numbers of compounds and the *Xeropus* oocyte is an amphibian not a mammalian system. Studies of the 10 electrophysiological properties of rodent ENaC in mammalian cell lines (HEK293 and MDCK) stably expressing the channel have been reported in the literature. While these studies used mammalian cell lines, channel function was assayed using tedious electrophysiological techniques. Such approaches do not lend themselves to high throughput screening of 15 compounds. Thus, there remains a need in the art for identification of salt taste enhancers amenable to high throughput screening.

[0008] The development of salt taste enhancers has been the focus of numerous prior scientific publications and patents. However, direct modulation of the ENaC sodium channel involved in salt taste perception is a 20 novel and unique approach to enhance human salty taste. Some examples of previously reported salt enhancing compounds and their properties are discussed below.

[0009] Some proteolzyed proteins, peptides, amino acids, and amino-acid esters reportedly function as salt enhancers (Tamura, et al, Argic. Biol. Chem.

(1989) 53(6): 1625-1633, 1989; US Patent 5,711,985). However, these agents require high concentrations, between 30-60 mM, and must be supplemented with hydrochloride acid to positively modulate salty taste. In addition, the cost and difficulty in synthesizing these compounds are 5 prohibitive for their large-scale commercial use as salt enhancers for the general population.

[0010] Choline chloride, an ammonium salt classified by the federal government as a GRAS (generally regarded as safe) compound, has been reported to function as a salt enhancer in humans and rodents. In humans, 10 choline chloride increases the saltiness of dilute salt solutions (less than 50 mM NaCl) by a factor of two and reportedly increases the preference or hedonic ratio of both cooked peas and Campbell's low salt tomato soup (Locke, et al, Physiology & Behavior (1994) 55(6): 1039-1046; US Patent 5,260,091; US Patent 5,260,049). However, similar to peptides and amino 15 acids described above, choline chloride requires significant concentrations (in the mM range) to enhance salty taste.

[0011] Derivatives of amiloride, which do not block ENaC function but instead block sodium-proton exchange, as well as chloride channel blockers, such as IAA-94 and anthranilic acid, reportedly increase fluid intake; an 20 indirect measurement of salt consumption, in a rodent model system (US Patent 5,260,091). However, the utility of these agents as human salt enhancers has not been reported.

[0012] Cetylpyridinium chloride (CPC) has been reported to increase amiloride-insensitive nerve responses to salt in rats and to enhance the

saltiness of low salt Campbell's tomato soup by 50% in humans when used at low concentrations (high uM range) (DeSimone, et al, J. Neurphysiol. (2001) 86: 2638-2641; US Patent 4,997,672). However, CPC is a detergent and based on its structure likely intercalates into lipid bilayers of cells and thereby

5 non-specifically activates salt taste cells by disrupting lipid homeostasis. Indeed, high concentrations of CPC (low mM range), above the critical micelle concentration, actually inhibit rat nerve responses to numerous salty compounds including sodium chloride, potassium chloride, and ammonium chloride, further substantiating that the reportedly observed CPC effects were

10 likely non-specific.

[0013] Trehalose, a disaccharide composed of two glucose molecules, reportedly increases the saltiness of sodium chloride solutions by 1.2 to 2-fold (US Patent 6,159,529). Similar to peptides and choline chloride, high levels (1.5-12%) of this sugar are required to enhance saltiness, suggesting that the

15 observed effects could be non-specific and attributable to taste cell volume changes (cell shrinkage) due to hyperosmolarity. In addition, the specificity of trehalose and other aforementioned salt enhancers to enhance salty taste and not modulate other tastes, including sweet, bitter, sour, and umami, was not addressed.

20 [0014] Alapyridaine, a derivative of the amino acid alanine that is formed as a by-product in heated sugar/amino acid mixtures, reportedly decreases the threshold for detecting sodium chloride 5-fold (Soldo, et al, Chemical Senses (2003) 28: 371-379, 2003; Ottlinger, et al, J. Agric Food Chem (2003) 51: 1035-1041, 2003). Alapyridaine, however, reportedly functions as a

general taste enhancer and decreases the detection thresholds for salt as well as sweet and umami tastes. In addition, the effect of alapyridaine on salt taste at higher, more physiologically-relevant, salt concentrations was not disclosed. Thus, the effects of alapyridaine may only surface when tasting low

5 salt concentrations near threshold detection levels.

[0015] The antibiotic novobiocin also reportedly enhances nerve responses to sodium chloride in rats (Feigin, et al, Am. J. Physiol. (1994) 266: C1165-C1172). However, disadvantageously novobiocin reportedly forms amiloride-insensitive cation-selective ion channels in lipid bilayers suggesting that this

10 agent pokes holes in cell membranes and, perhaps similar to CPC, non-specifically increase taste cell activity. The effect of novobiocin on human salt taste perception has not been reported.

[0016] Bretylium tosylate, an anti-fibrillary drug that modulates adrenergic and muscarinic receptors, has been reported to specifically potentiate salt

15 taste in rodents and humans without affecting sweet, sour, or bitter taste (Schiffman, et al, Physiology & Behavior (1986) 36: 1129-1137). However, a significant disadvantage of bretylium tosylate, separate from the relatively high concentrations required to positively modulate salty taste (mM range), is that

20 the compound is a therapeutic used to treat cardiac patients. Consequently this compound would be unsuitable for use in the general population.

[0017] Glybenclamide, an inhibitor of members of the ATP-binding cassette (ABC) protein superfamily, including the cystic fibrosis transmembrane conductance regulator and the sulfonylurea receptor, reportedly increases amiloride-sensitive ENaC sodium current by doubling the open probability of

individual ENaC channels (Chraibi, et al, The Journal of Pharmacology and Experimental Therapeutics (1999) 290: 341-347, 1999; Schnizler, et al, Biochimica et Biophysica Acta (2003) 1609: 170-176). However, because

5 Glybenclamide modulates ABC protein function, it is probable that Glybenclamide effects are due to indirect modulation of ENaC activity by ABC proteins and not attributable to direct modulation of ENaC channel function.

In addition, glybenclamide has not been demonstrated to enhance human salt taste perception nor has glybenclamide been suggested as a salt taste enhancer.

10 [0018] Thus, based on the foregoing, it is evident that improved methods for identifying compounds that specifically modulate ENaC and salty taste are needed as are improved salty taste modulators. Preferably, such methods will comprise high or medium throughput methods and will screen for compounds having a direct effect on human ENaC function.

15 **Summary of the Invention**

[0019] The present invention obviates the problems of the prior art, relating to assays for identifying compounds that modulate ENaC. Specifically, the present invention provides cell-based assays that utilize recombinant host cells, preferably mammalian cells or oocytes that express a functional human

20 ENaC to identify compounds that modulate ENaC and consequently salty taste. More specifically, the present invention provides oocyte and mammalian cell-based assays, preferably high or medium throughput, for the profiling and screening of a sodium channel, more particularly an amiloride-sensitive epithelial sodium channel (ENaC), which assays optionally may

include the addition of a compound that partially or totally inhibits ENaC function, preferably amiloride or an amiloride derivative such as phenamil. It has been found that the use of phenamil in particular enhances signal intensity during assays, preferably high or medium throughput assays for 5 identifying compounds that modulate (enhance or inhibit) ENaC function. Such methods can be used to functionally characterize ENaC activity or to identify compounds that either enhance or block salty taste perception (herein referred to as salty taste modulators).

[0020] Accordingly, in a first aspect the invention provides recombinant 10 host cells, preferably mammalian cells or amphibian oocytes that express a functional hENaC. In a preferred embodiment these cells will transiently or stably express all three subunits of hENaC (alpha or delta, beta and gamma), or transiently or stably express one or more subunits or functional chimeras, variants or fragments thereof. Mammalian cells suitable for use in the 15 invention encompass any mammalian cell capable of expressing a functional hENaC, including by way of example COS, CHO, MDCK, HEK293, HEK293T, NIH3T3, Swiss3T3 and BHK cells. However, in the preferred embodiment the invention provides HEK293T cells that express a functional hENaC. Oocytes useful in the invention preferably include amphibian oocytes, e.g., *Xenopus* 20 oocytes.

[0021] In a second aspect, the invention provides cell-based assays that utilize mammalian cells or amphibian oocytes that express a functional ENaC, preferably hENaC, to identify compounds, including e.g., small organic molecules, antibodies, peptides, cyclic peptides, lipids and nucleic acids that

enhance or block ENaC function. Preferably, these assays will include the addition of known ENaC inhibitor at a concentration that partially inhibits ENaC function prior to addition of use of one or more putative ENaC modulatory compounds.

- 5 [0022] Preferably the assay will comprise a mammalian or oocyte cell-based assay, preferably high or medium throughput, for the profiling and screening of putative modulators of an epithelial sodium channel (ENaC) comprising: (i) contacting a test cell expressing an ENaC loaded with a membrane potential fluorescent dye or a sodium-sensitive fluorescent dye
- 10 with at least one putative modulator compound in the presence of a buffer containing sodium; (ii) prior to the addition of said at least one putative modulator compound, contacting said host cell with a compound that is known to inhibit ENaC function, at a concentration whereby ENaC function is at least partially inhibited, preferably an amiloride derivative such as phenamil; and
- 15 (iii) monitoring changes in fluorescence of the membrane potential dye or sodium-sensitive dye in cells contacted with the putative modulator plus sodium after addition of the known ENaC inhibitor compound compared to the change in fluorescence of the membrane potential dye or sodium-sensitive dye for cells contacted with sodium alone to determine the extent of ENaC
- 20 modulation.

[0023] In another preferred aspect of the invention, a method for monitoring the activity of an epithelial sodium channel (ENaC) is provided comprising: (i) providing test cells, e.g., mammalian cells transfected or transformed with a functional ENaC; (ii) seeding the test cell in the well of a

multi-well plate and incubating for a time sufficient to reach at least about 70% confluence; (iii) dye-loading the seeded test cells with a membrane potential fluorescent dye or sodium-sensitive fluorescent dye in the well of the multi-well plate; (iv) contacting the dye-loaded test cell with at least one putative 5 modulatory compound in the well of the multi-well plate; (v) prior to the addition of said at least one putative modulatory compound, further contacting said host cell with a compound that partially inhibits ENaC function, e.g., an amiloride derivative such as phenamil; and (vi) monitoring any changes in fluorescence using a fluorescence plate reader.

10 [0024] In another preferred embodiment of the invention (i) suitable cells, e.g., HEK293T cells or another mammalian cell line are transformed, transfected with DNA sequences encoding subunits necessary to produce a functional human ENaC; (ii) the cells are seeded onto a multi-well plates, e.g., 384 well plates, preferably to about 80% confluence; (iii) the seeded test cells 15 are loaded with a membrane potential sensitive dye such as CC2-DMPVE or DiSBAC2(3); (iv) the dye-loaded cells are then contacted with at least one putative ENaC modulatory compound; (v) the dye-loaded cells are preferably further contacted prior to contacting with said at least one ENaC modulatory compound with an amount of at least one known ENaC inhibitor at a 20 concentration that results in at least partial ENaC inhibition; and (iv) monitoring changes in cell fluorescence using a voltage intensity plate reader e.g., VIPRII (Aurora Biosciences).

[0025] In yet another aspect of the invention, a method for identifying a salty taste modulatory compound is provided comprising: (i) providing test

cells transfected, transformed with a functional human ENaC; (ii) seeding the test cell in the well of a multi-well plate and incubating for a time sufficient to reach at least about 70% confluence more preferably to about 80%, confluence; (iii) dye-loading the seeded test cells with a membrane potential

5 dye in the well of the multi-well plate; (iv) contacting the dye-loaded test cells with at least one putative modulatory compound in the well of the multi-well plate; (v) preferably prior to the addition of said at least one putative modulatory compound further contacting the dye-loaded test cell with a known ENaC inhibitor compound at a concentration that at least partially inhibit ENaC

10 function, e.g., an amiloride derivative such as phenamil; (vi) monitoring any changes in fluorescence of the membrane potential dye due to modulator/ENaC interactions using a fluorescence plate reader; and (vii) identifying the at least one putative modulator as a salty taste modulating compound based on the monitored changes in fluorescence.

15 [0026] In yet another preferred embodiment of the invention (i) suitable cells, e.g., HEK293T cells are transformed or transfected with DNA sequences encoding subunits necessary to produce a functional human ENaC; (ii) the cells are seeded onto multi-well plates, e.g., 384 well plates, preferably to about 80% confluence; (iii) the seeded test cells are loaded with a membrane

20 potential sensitive dye such as CC2-DMPVE or DiSBAC2(3); (iv) the dye-loaded cells are then contacted with at least one putative ENaC modulatory compound; (v) preferably prior thereto the dye-loaded cells are contacted with a compound known to inhibit ENaC function, e.g., an amiloride derivative such as phenamil at a concentration that at least partially inhibits ENaC function;

25 (vi) changes in cell fluorescence are monitored using a voltage intensity plate

reader e.g., VIPRII (Aurora Biosciences); and (vii) compounds that modulate salty taste are selected based on a change in fluorescence intensity.

[0027] In yet another preferred embodiment of the invention, electrophysiological assays, preferably two-electrode voltage clamp assays 5 are provided wherein human ENaC modulatory compounds are identified based on their effect (inhibitory or enhancing) on macroscopic electrical current in oocytes, preferably amphibian oocytes (frog), that express a functional human ENaC sodium channel. These assays are an improvement over some other cell-based assays for identifying ENaC modulators, in part 10 because oocytes express few endogenous ion channels; consequently the oocyte expression system advantageously allows direct measurement of ENaC sodium channel current with little or no background.

[0028] In another preferred embodiment of the invention, these electrophysiological assays will further contact said oocytes with at least a 15 partial inhibitor of ENaC, e.g., amiloride or an amiloride derivative as a control or in order to enhance measurable changes in sodium channel cell current.

[0029] In yet another preferred embodiment of the invention, frog oocytes that functionally express a human ENaC sodium channel are provided which express human ENaC alpha, beta and gamma or delta, beta and gamma 20 subunits.

[0030] In another preferred embodiment of the invention, the ENaC used in cell-based assays according to the invention can be composed of naturally occurring human ENaC subunits, one or more alternatively spliced human

ENaC subunits, or a functional variant thereof. Alternatively, the ENaC can be composed of at least the alpha subunit of a naturally occurring human ENaC, or an alternatively spliced version thereof. In another embodiment, a delta subunit (such as Genebank accession U38254; see *J Biol Chem*, 5 270(46):27411-4 (1995)) or a variant thereof can substitute for the alpha subunit.

[0031] Preferably, these subunits are encoded by SEQ ID: NO.: 1, 2, 3 and 7 disclosed infra. These and other aspects of the invention will become apparent to one of skill in the art from the following detailed description, 10 drawings, and claims.

#### **Detailed Description of the Drawings**

[0032] Figure 1 illustrates the functional expression of hENaC resulting in a sodium dependent amiloride sensitive fluorescence change. Transfection of HEK293T cells with varying 1:1:1 ratios of  $\alpha$ ,  $\beta$ , and  $\gamma$ , subunit plasmids of 15 human kidney ENaC results in a  $\text{Na}^+$  dependent amiloride sensitive voltage change, as compared to mock transfected cells. A, B, C, and D were transfected with 111:1 ratios of  $\alpha$ ,  $\beta$ , and  $\gamma$  plasmid at absolute levels of 4.4.1. and 0.25 respectively. E and F were mock transfected with Beta-gal and pUC. Transfection efficiency was approximately 40% and cell density was 20 approximately 70%. All traces are from a single plate with A (n=4), B, C, D, E (n=12), and F (n=8).

[0033] Figure 2 illustrates the  $\text{NaCl}$  dose response relationship of HEK293T cells expressing hENaC  $\alpha$ ,  $\beta$ , and  $\gamma$ .

[0034] Figure 3 illustrates the amiloride dose response relationship of HEK293T cells expressing hENaC  $\alpha$ ,  $\beta$ , and  $\gamma$  treated with 50 mM NaCl.

[0035] Figures 4 illustrates the NaCl dose response relationship of HEK293T cells expressing ENaC using a voltage imaging plate reader (VIPR).

5 HEK293T cells were transfected with ENaC subunits expression plasmids (ENaC) or a carrier plasmid (Mock). 24 hours later cells were loaded with a membrane potential dyes and changes in cell fluorescence in response to Na<sup>+</sup> stimulation was monitored on VIPRII (Aurora Biosciences). Only cells expressing ENaC exhibited a change in response to increases in Na<sup>+</sup>

10 concentration.

[0036] Figure 5 also illustrates the NaCl dose response relationship of HEK293T cells expressing human ENaC. HEK293T cells were transfected with ENaC subunits expression plasmids (ENaC) 24 hours later cells were

15 loaded with a membrane potential dyes and changes in cell fluorescence in response to Na<sup>+</sup> stimulation was monitored on VIPRII (Aurora Biosciences).

Phenamil, an ENaC antagonist, inhibited Na<sup>+</sup>-induced changes in fluorescence. Conversely, the Compound "X", an ENaC enhancer, increased the Na<sup>+</sup>-induced changes in fluorescence and this effect is inhibited by Phenamil.

20 [0037] Figure 6 shows the effect of increasing concentrations of Phenamil on ENaC activity. The blue trace: inhibition of ENaC activity by Phenamil. Red trace: inhibition of ENaC activity by Phenamil in the presence of 100  $\mu$ M compound 478354, an ENaC enhancer. The black box contains data showing compound 478354's effect in the absence of Phenamil. The yellow box

contains data showing enhanced 478354 effects by the presence of increasing concentrations of Phenamil.

[0038] Figure 7A. Distribution of Z' in the absence of Phenamil. "Z" is defined as:  $1 - ((3X \text{ standard deviation of ENaC responds to 3X standard deviation of ENaC response in the presence of compound 478354}) / (\text{mean ENaC activity in the presence of 478354} - \text{mean ENaC activity}))$ . Most Z' values are less than 0 indicating that, when used in the high control, 479354 can not provide a meaningful assay window.

[0039] Figure 7B. Distribution of Z' in the presence of  $0.5\mu\text{M}$  Phenamil.  $Z^1$  is defined as:  $1 - ((3X \text{ standard deviation of ENaC response} + 3X \text{ standard deviation of ENaC response in the presence of 478354}) / (\text{mean ENaC activity in the presence of 478354} - \text{mean ENaC activity}))$ . Most  $Z^1$  values are  $> 0$  and  $\leq 1$  indicating that as the high control, 478354 can provide a meaningful assay window in the presence of Phenamil.

[0040] Figure 8 illustrates an example of screening oocytes injected with human ENaC cRNA for compounds that increase ENaC activity. For each compound screened, a % enhancement factor is calculated. This value corresponds to the magnitude of the current change due to compound divided by the magnitude of the current change due to amiloride multiplied by  $-100\%$ .  
In this example, two compounds are screened in succession in 7, out of a possible maximum 8, oocytes voltage clamped to  $-60$  mV in the OpusXpress system. All 7 oocytes express ENaC, as evidenced by the inhibitory effect of amiloride on measured oocyte currents.

[0041] Figure 9 illustrates an example of how the % enhancement factor is calculated for each oocyte injected with human ENaC cRNA. % enhancement factors are determined for each compound screened, averaged, and standard deviations determined. In this case, compound 1 and compound 2 5 correspond to the compounds screened in cells numbered 2 though 8 in Figure 8.

[0042] Figure 10 illustrates an example of screening oocytes not injected with human ENaC cRNA. Compounds have no effect on the activity of ion channels expressed endogenously in the oocyte membrane, illustrating that 10 compound activity is ENaC-dependent and attributable to increased macroscopic sodium current flowing through ENaC channels. Also the figure shows that amiloride has no effect on uninjected oocytes due to the absence of ENaC sodium channel expression.

[0043] Figure 11 illustrates an example of I/V curves in oocytes injected 15 with human ENaC cRNA or uninjected oocytes in the presence and absence of compound. In injected eggs, the compound increases the slope of the I/V curve, whereas in uninjected oocytes the compound has no effect on the slope of the I/V curve (i.e. the curves in the presence and absence of compound are identical).

[0044] Figure 12 illustrates an example of an amiloride competition 20 experiment. In oocytes injected with human ENaC cRNA, co-application of amiloride plus compound does not enhance sodium currents flowing through ENaC channels. This indicates that compounds are working directly on the

ENaC channel; when ENaC channels are closed due to amiloride, compounds cannot enhance ENaC function.

[0045] Figure 13 illustrates an example of dose-response curves for 2 compounds in oocytes injected with human ENaC cRNA. Compound A is less 5 potent than compound B as evidenced by its larger EC50 (5.4 uM with compound A compared to 0.47 uM with compound B) and right-shifted dose-response curve.

[0046] Figure 14 schematically illustrates a set of experiments used to examine the effect of compounds on human ENaC activity in the oocyte 10 expression system using the two-electrode voltage clamp (TEVC) technique.

#### **Detailed Description of the Invention**

[0047] The present invention provides assay systems that comprise test cells, preferably recombinant mammalian cells or amphibian oocytes, that express a functional hENaC as well as mammalian cell-based and amphibian 15 oocyte cell-based assays, preferably high or medium throughput, for the profiling and screening of an epithelial sodium channel (ENaC). More specifically, the invention provides human cell lines, e.g., HEK293T cells, and amphibian oocytes, that express the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits of hENaC that can be used in cell-based assays to screen for ENaC modulators. Also the 20 invention provides mammalian cells and amphibian oocytes that express a functional ENaC comprised of delta, beta and gamma subunits for use in functionally characterizing ENaC activity, and to identify compounds that either enhance or block salty taste perception (herein referred to as salty taste

modulators). These compounds can be used as ingredients in foods, medicinals and beverages to enhance, modulate, inhibit or block salty taste.

[0048] However, prior to discussing the invention in more detail the following definitions are provided. It should be otherwise understood that the 5 technical terms and phrases have their ordinary meaning, as they would be construed by use of ordinary skill in the art.

### **Definitions**

[0049] The term 'salty taste" or "salty taste perception" as used herein refers to a subject's perception or response to salt taste stimuli. As discussed 10 above, it is believed that hENaC is involved in salty taste perception, in particular salts that elicit "a salty taste" in human subjects. Such stimuli include compounds such as NaCl that elicit a response in functional ENaCs, preferably hENaC.

[0050] The terms "ENaC" subunit protein or a fragment thereof, or a 15 nucleic acid encoding one of three subunits of "ENaC" protein or a fragment thereof refer to nucleic acids and polypeptides, polymorphic variants, alleles, mutants, and interspecies homologues that: (1) have an amino acid sequence that has greater than about 80% amino acid sequence identity, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater 20 amino acid sequence identity, preferably over a region of over a region of at least about 25, 50, 100, 200, or 500, or more amino acids, to an amino acid sequence encoded by the nucleic acid sequence contained in SEQ ID NO:1; 2 or 3; or (2) specifically bind to antibodies, e.g., polyclonal antibodies, raised against an immunogen comprising an amino acid sequence encoded by SEQ

ID NO:1, 2, or 7 or immunogenic fragments thereof, and conservatively modified variants thereof; or (3) specifically hybridize under stringent hybridization conditions to an anti-sense strand corresponding to a nucleic acid sequence encoding an ENaC protein, e.g., SEQ ID NO:1, 2, 3 or 7 or 5 their complements, and conservatively modified variants thereof; or (4) have a nucleic acid sequence that has greater than about 80% sequence identity, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or higher nucleotide sequence identity, preferably over a region of at least about 10 25, 50, 100, 200, 500, 1000, or more nucleotides, to SEQ ID NO:1, 2, 3 or 7 or their complements, or (5) is functionally equivalent to the hENaC described herein in a sodium conductance assay when expressed in a HEK cell and tested by using two electrode whole cell electrophysiology or by the change in fluorescence of a membrane potential dye in response to sodium or lithium.

[0051] Functionally equivalent ENaC proteins include ENaC subunits with 15 primary sequences different than those identified infra, but which possess an equivalent function as determined by functional assays, e.g., sodium conductance assays as described infra.

[0052] "Determining the functional effect" refers to assaying the effect of a compound that increases or decreases a parameter that is indirectly or 20 directly under the influence of an ENaC polypeptide e.g., functional, physical and chemical effects. Such functional effects include, but are not limited to, changes in ion flux, membrane potential, current amplitude, and voltage gating, as well as other biological effects such as changes in gene expression of any marker genes, and the like. The ion flux can include any

ion that passes through the channel, e.g., sodium or lithium and analogs thereof such as radioisotopes. Such functional effects can be measured by any means known to those skilled in the art, e.g., by the use of two electrode electrophysiology or voltage-sensitive dyes, or by measuring changes in 5 parameters such as spectroscopic characteristics (e.g., fluorescence, absorbance, refractive index), hydrodynamic (e.g., shape), chromatographic, or solubility properties. Preferably ENaC function will be evaluated by using two electrode whole cell electrophysiology or by monitoring the change in fluorescence of a membrane potential dye in response to sodium or lithium.

10 [0053] "Inhibitors", "activators", and "modulators" of ENaC polynucleotide and polypeptide sequences are used to refer to activating, inhibitory, or modulating molecules identified using cell-based assays of ENaC polynucleotide and polypeptide sequences. Inhibitors are compounds that, e.g., bind to, partially or totally block activity, decrease, prevent, delay 15 activation, inactivate, desensitize, or down regulate the activity or expression of ENaC proteins, e.g., antagonists.

20 [0054] "Activators" are compounds that increase, open, activate, facilitate, enhance activation, sensitize, agonize, or up regulate ENaC protein activity. Inhibitors, activators, or modulators also include genetically modified versions of ENaC proteins, e.g., versions with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, peptides, cyclic peptides, nucleic acids, antibodies, antisense molecules, ribozymes, small organic molecules and the like. Such assays for inhibitors and activators include, e.g., expressing ENaC protein in cells, cell extracts, or cell

membranes, applying putative modulator compounds, and optionally prior thereto contacting said ENaC protein with a known ENaC inhibitor at a concentration that results in partial ENaC inhibitors and then determining the functional effects of the rotation compound on activity, as described above.

5 [0055] Samples or assays comprising ENaC proteins that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of activation, inhibition or modulation. In one embodiment of the assay, compounds are tested for their effect on the response of cells provided with a

10 suboptimal sodium concentration. Control cells, treated with the suboptimal concentration of sodium but lacking a compound, typically exhibit a 10-20% of the maximal response. Compounds that increase the response of the suboptimal sodium concentration above the 10-20% level are putative ENaC enhancers. In contrast, compounds that reduce the response to below 10%

15 are putative ENaC enhancers.

[0056] The term "test compound" or "test candidate" or "modulator" or grammatical equivalents thereof as used herein describes any molecule, either naturally occurring or synthetic, e.g., protein, oligopeptide (e.g., from about 5 to about 25 amino acids in length, preferably from about 10 to 20 or

20 12 to 18 amino acids in length, preferably 12, 15, or 18 amino acids in length), small organic molecule, polysaccharide, lipid (e.g., a sphingolipid), fatty acid, polynucleotide, oligonucleotide, etc., to be tested for the capacity to modulate ENaC activity. The test compound can be in the form of a library of test compounds, such as a combinatorial or randomized library that provides a

sufficient range of diversity. Test compounds are optionally linked to a fusion partner, e.g., targeting compounds, rescue compounds, dimerization compounds, stabilizing compounds, addressable compounds, and other functional moieties. Conventionally, new chemical entities with useful properties are generated by identifying a test compound (called a "lead compound") with some desirable property or activity, e.g., enhancing activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Preferably, high throughput screening (HTS) methods are employed for such an analysis.

10 [0057] A "small organic molecule" refers to an organic molecule, either naturally occurring or synthetic, that has a molecular weight of more than about 50 daltons and less than about 2500 daltons, preferably less than about 2000 daltons, preferably between about 100 to about 1000 daltons, more preferably between about 200 to about 500 daltons.

15 [0058] "Biological sample" includes sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes. Such samples include blood, sputum, tissue, cultured cells, e.g., primary cultures, explants, and transformed cells, stool, urine, etc. A biological sample is typically obtained from a eukaryotic organism, most preferably a mammal  
20 such as a primate e.g., chimpanzee or human; cow; dog; cat; a rodent, e.g., guinea pig, rat, mouse; rabbit; or a bird; reptile; or fish.

[0059] "Compound that inhibits ENaC activity" refers to a compound which inhibits sodium channel activity, preferably reversibly when this compound is contacted with a functional ENaC. Preferred examples of such compounds

are amiloride and amiloride derivatives such as Phenamil, benzamil, 3<sup>1</sup>, 4<sup>1</sup>– dichlorobenzamil, ethylisopropylamiloride; 5-(N-4-chlorobenzyl)-2<sup>1</sup>, 4<sup>1</sup> dimethyl-benzamil, 5-(N-methyl-N-guanidinocarbonylmethyl) amiloride; 5-(N, N-hexa-myethylene) amiloride; 5-(N-ethyl-N-isopropyl) amiloride (EIPA); 5-(N- 5 4-chloro-benzyl) 2<sup>1</sup>, 4<sup>1</sup> dimethylbenzamil, 2<sup>1</sup>, 4<sup>1</sup>–dimethylbenzamil; 2<sup>1</sup>, 3<sup>1</sup>– benzo-benzamil; and the like.

[0060] “Amiloride derivative” refers to a compound having a structure similar to amiloride which inhibits ENaC function. Typically such derivatives are substituted on the guanidine substituent (e.g., Phenamil) or on the 5-N 10 position (e.g., ethylisopropylamiloride).

[0061] The terms “identical” or percent “identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 80% identity, 15 preferably 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region (e.g., nucleotide sequences SEQ ID NO: 1, 2, 3 or 7), when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters 20 described below, or by manual alignment and visual inspection (see, e.g., NCBI web site ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) or the like). Such sequences are then said to be “substantially identical.” This definition also refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have

substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

5 [0062] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default  
10 program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

15 [0063] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for  
20 comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA*

85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

[0064] A preferred example of an algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990),

10 respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by  
15 identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer  
20 HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid  
25 sequences, a scoring matrix is used to calculate the cumulative score.

Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is 5 reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, 10 and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0065] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The 15 terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

[0066] The term "amino acid" refers to naturally occurring and synthetic 20 amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline,  $\gamma$ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to

compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an  $\alpha$  carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have

5 modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but those functions in a manner similar to a naturally occurring amino acid.

10 [0067] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0068] "Conservatively modified variants" applies to both amino acid and 15 nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number 20 of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations,"

which are one species of conservatively modified variations. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon 5 for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence with respect to the expression product, but not with respect to actual probe sequences.

10 [0069] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the 15 substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologous, and alleles of the invention.

20 [0070] The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S),

Threonine (T); and 8) Cysteine (C), Methionine (M) (see, e.g., Creighton, *Proteins* (1984)). As noted previously, the invention embraces cells that express ENaC subunit polypeptides having primary sequences different than those disclosed in the subject application that are functionally equivalent in appropriate assays, e.g., using whole cell sodium conductance assays described in detail infra.

5 [0071] Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, see, e.g., Alberts *et al.*, *Molecular Biology of the Cell* (3<sup>rd</sup> ed., 1994) and Cantor and Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered three-dimensional structures within a polypeptide. These structures are commonly known as domains, e.g., transmembrane

10 domains pore domains; and cytoplasmic tail domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 15 to 350 amino acids long. Exemplary domains include extracellular domains, transmembrane domains, and cytoplasmic domains. Typical domains are made up of sections of lesser organization such as stretches of

15  $\alpha$ -sheet and  $\beta$ -helices. "Tertiary structure" refers to the complete three-dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units. Anisotropic terms are also known as

20 energy terms.

[0072] A particular nucleic acid sequence also implicitly encompasses "splice variants." Similarly, a particular protein encoded by a nucleic acid implicitly encompasses any protein encoded by a splice variant of that nucleic acid. "Splice variants," as the name suggests, are products of alternative

5 splicing of a gene. After transcription, an initial nucleic acid transcript may be spliced such that different (alternate) nucleic acid splice products encode different polypeptides. Mechanisms for the production of splice variants vary, but include alternate splicing of exons. Alternatively polypeptides derived from the same nucleic acid by read-through transcription are also encompassed by  
10 this definition. Any products of a splicing reaction, including recombinant forms of the splice products, are included in this definition.

[0073] ENaC nucleic acid sequences also include single nucleotide polymorphisms which encode ENaC subunits that are functionally equivalent to the ENaC polypeptides disclosed herein when assayed using appropriate  
15 assays, in the sodium conductance assays described herein.

[0074] Membrane potential dyes or voltage-sensitive dyes refer to a molecule or combinations of molecules that change fluorescent properties upon membrane depolarization. These dyes can be used to detect the changes in activity of an ion channel such as ENaC expressed in a cell.

20 [0075] A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include  $^{32}P$ , fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made

detectable, e.g., by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

[0076] The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or

5 vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under 10 expressed, or not expressed at all. In the present invention this typically refers to cells that have been transfected with nucleic acid sequences that encode one or more ENaC subunits.

[0077] The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more

15 subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the 20 protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein). The term "heterologous" when used with reference to cellular expression of a gene, cDNA, mRNA or protein indicates that the gene, cDNA, mRNA, or protein is

not normally expressed in the cell or is from another species than the original source of the cells.

[0078] The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. The wash and hybridization steps are generally carried out for ½, 1, 2, 5, 13, 15, 30, 60 or more minutes, and more typically for about 30 seconds to 2 minutes.

[0079] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the 5 genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will 10 readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous reference, e.g., and *Current Protocols in Molecular Biology*, ed. Ausubel, et al.

[0080] For PCR, a temperature of about 36°C is typical for low stringency 15 amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low 20 stringency amplifications include a denaturation phase of 90°C - 95°C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, e.g., in Innis et al. (1990) *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y.).

[0081] "Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes,

5 as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding.

10 [0082] Particularly, such an antibody includes one which specifically binds to an ENaC disclosed herein, or a mixture of antibodies that specifically bind such ENaC polypeptides.

[0083] The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein

15 or peptide, refers to a binding reaction that is determinative of the presence of the protein, often in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and more typically more than 10 to 100 times background. Specific binding to an  
20 antibody under such conditions requires an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to ENaC subunit proteins, e.g., the ENaC alpha, beta, gamma or delta subunits as encoded by SEQ ID NO:1, 2, 3, or 7, polymorphic variants, alleles, orthologs, and conservatively modified variants, or splice variants, or

portions thereof, can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with ENaC subunit proteins i.e., ENaC alpha, beta, gamma or delta subunits, e.g., those having the amino acid sequences contained in SEQ ID NO.: 4, 5, 6 or 8, and not with other proteins.

- 5 This selection may be achieved by subtracting out antibodies that cross-react with other molecules. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow &
- 10 Lane, *Antibodies, A Laboratory Manual* (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

#### **Assays for proteins that modulate ENaC activity**

[0084] High or medium throughput functional genomics assays can be used to identify modulators of ENaC which block, inhibit, modulate or enhance salty taste. Such assays can, e.g., monitor changes in cell surface marker expression, changes in intracellular ions, or changes in membrane currents using either cell lines or primary cells or oocytes. Typically, the cells are contacted with a cDNA or a random peptide library (encoded by nucleic acids). The cDNA library can comprise sense, antisense, full length, and truncated cDNAs. The peptide library is encoded by nucleic acids. The effect of the cDNA or peptide library on the phenotype of the cells is then monitored, using an assay as described above. The effect of the cDNA or peptide can be validated and distinguished from somatic mutations, using, e.g., regulatable expression of the nucleic acid such as expression from a tetracycline

promoter. cDNAs and nucleic acids encoding peptides can be rescued using techniques known to those of skill in the art, e.g., using a sequence tag.

[0085] Proteins interacting with the peptide or with the protein encoded by the cDNA (e.g., SEQ ID NO: 1, 2, or 7) can be isolated using a yeast two-

5 hybrid system, mammalian two hybrid system, or phage display screen, etc.

Targets so identified can be further used as bait in these assays to identify additional components that may interact with the ENaC channel which members are also targets for drug development (see, e.g., Fields *et al.*,

*Nature* 340:245 (1989); Vasavada *et al.*, *Proc. Nat'l Acad. Sci. USA* 88:10686

10 (1991); Fearon *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:7958 (1992); Dang *et al.*,

*Mol. Cell. Biol.* 11:954 (1991); Chien *et al.*, *Proc. Nat'l Acad. Sci. USA* 9578

(1991); and U.S. Patent Nos. 5,283,173, 5,667,973, 5,468,614, 5,525,490,

and 5,637,463).

[0086] Suitable cells and cell lines that express ENaC proteins include, by

15 way of example, kidney epithelial cells, lung epithelial cells, taste epithelial cells and other mammalian epithelial cells, preferably human., and oocytes, preferably amphibian oocytes, most preferably *Xenopus* oocytes.

#### **Isolation of nucleic acids encoding ENaC proteins**

[0087] This invention relies, in part, on routine techniques in the field of

20 recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook and Russell, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

[0088] Nucleic acids that encode ENaC subunits, polymorphic variants, orthologs, and alleles that are substantially identical to an amino acid sequence encoded by SEQ ID NO: 1, 2, 3 or 7 as well as other ENaC family members, can be isolated using ENaC nucleic acid probes and oligonucleotides under stringent hybridization conditions, by screening libraries. Alternatively, expression libraries can be used to clone ENaC subunit protein, polymorphic variants, orthologs, and alleles by detecting expressed homologous immunologically with antisera or purified antibodies made against human ENaC or portions thereof.

10 [0089] To make a cDNA library, one should choose a source that is rich in ENaC RNA. The mRNA is then made into cDNA using reverse transcriptase, ligated into a recombinant vector, and transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known (see, e.g., Gubler & Hoffman, *Gene* 25:263-269 15 (1983); Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

[0090] For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. 20 These vectors and phage are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA.*, 72:3961-3965 (1975).

[0091] Alternatively, ENaC cRNA encoding human ENaC subunits may be generated from  $\alpha$ ,  $\beta$ ,  $\gamma$  or  $\Delta$  human ENaC DNA plasmids using T7 RNA polymers to transcribe cRNA in vitro from DNA linearized with appropriate restriction enzymes and the resultant cRNA microinjected into suitable cells, e.g., oocytes, preferably frog oocytes.

[0092] An alternative method of isolating ENaC subunit nucleic acid and its orthologs, alleles, mutants, polymorphic variants, and conservatively modified variants combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA template (see U.S. Patents 4,683,195 and 10 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid sequences of human ENaC directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Degenerate oligonucleotides can be designed to amplify ENaC 15 homologs using the sequences provided herein. Restriction endonuclease sites can be incorporated into the primers. Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of ENaC encoding 20 mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can be purified from agarose gels and cloned into an appropriate vector.

[0093] Gene expression of ENaC subunits can also be analyzed by techniques known in the art, e.g., reverse transcription and amplification of

mRNA, isolation of total RNA or poly A<sup>+</sup> RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, high density polynucleotide array technology, e.g., and the like.

[0094] Nucleic acids encoding ENaC subunit proteins can be used with 5 high-density oligonucleotide array technology (e.g., GeneChip<sup>TM</sup>) to identify ENaC protein, orthologs, alleles, conservatively modified variants, and polymorphic variants in this invention. In the case where the homologs being identified are linked to modulation of T cell activation and migration, they can be used with GeneChip<sup>TM</sup> as a diagnostic tool in detecting the disease in a 10 biological sample, see, e.g., Gunthand *et al.*, *AIDS Res. Hum. Retroviruses* 14: 869-876 (1998); Kozal *et al.*, *Nat. Med.* 2:753-759 (1996); Matson *et al.*, *Anal. Biochem.* 224:110-106 (1995); Lockhart *et al.*, *Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al.*, *Genome Res.* 8:435-448 (1998); and Hacia *et al.*, *Nucleic Acids Res.* 26:3865-3866 (1998).

15 [0095] The genes encoding ENaC subunits preferably human ENaC subunits are typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryotic vectors, e.g., plasmids, or shuttle vectors.

20

#### 1. Expression in prokaryotes and eukaryotes

[0096] To obtain high level expression of a cloned gene, such as those cDNAs encoding hENaC subunit, one typically subclones the hENaC subunit nucleic acid sequence into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for

a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook *et al.*, and Ausubel *et al, supra*. Bacterial expression systems for expressing the ENaC subunit protein are available in, 5 e.g., *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, *Xenopus* oocytes and insect cells are well known in the art and are also commercially available.

10 [0097] In a preferred embodiment of the invention, an oocyte expression system is used to express a functional human ENaC and to examine the effects of specific compounds on sodium transport through ENaC channels. The *Xenopus* oocyte expression system has previously been used for the expression of ion channels, including ENaC, and in functional studies (Dascal, 15 CRC Crit. Rev. Biotech. (1987) 22(4):317-387; Wagner, *et al.*, *Cellular Physiology and Biochemistry* (2000) 10:1-12; and Canessa *et al.*, *Nature* (1994) 367:463-467). In still another embodiment retroviral expression systems may be used in the invention. In another embodiment transient expression systems may be utilized with plasmid-based vectors that are 20 commercially available such as pcDNA 3 and derivatives thereof

[0098] Selection of the promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site, as it is from the transcription start site in

its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[0099] In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional 5 elements required for the expression of the ENaC subunit encoding nucleic acid in host cells. A typical expression cassette thus contains at least one promoter operably linked to a nucleic acid sequence encoding a ENaC subunit(s) and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. Additional elements of the 10 cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor site.

[00100] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be 15 obtained from the same gene as the promoter sequence or may be obtained from different genes.

[00101] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. 20 Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as MBP, GST, and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc. Sequence tags may be included in an expression cassette for nucleic acid rescue.

Markers such as fluorescent proteins, green or red fluorescent protein,  $\beta$ -gal, CAT, and the like can be included in the vectors as markers for vector transduction.

[00102] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, retroviral vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A<sup>+</sup>, pMTO10/A<sup>+</sup>, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the CMV promoter, SV40 early promoter, SV40 late promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[00103] Expression of proteins from eukaryotic vectors can be also regulated using inducible promoters. With inducible promoters, expression levels are tied to the concentration of inducing agents, such as tetracycline or ecdysone, by the incorporation of response elements for these agents into the promoter. Generally, high level expression is obtained from inducible promoters only in the presence of the inducing agent; basal expression levels are minimal.

[00104] In one embodiment, the vectors of the invention may have a regulatable promoter, e.g., tet-regulated systems and the RU-486 system (see, e.g., Gossen & Bujard, *PNAS* 89:5547 (1992); Oligino *et al.*, *Gene Ther.* 5:491-496 (1998); Wang *et al.*, *Gene Ther.* 4:432-441 (1997); Neering *et al.*,

*Blood* 88:1147-1155 (1996); and Rendahl *et al.*, *Nat. Biotechnol.* 16:757-761

(1998)). These impart small molecule control on the expression of the candidate target nucleic acids. This beneficial feature can be used to determine that a desired phenotype is caused by a transfected cDNA rather than a somatic mutation.

5 [0097] Some expression systems have markers that provide gene amplification such as thymidine kinase and dihydrofolate reductase.

Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with a 10 ENaC encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

15 [00105] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids,

and unique restriction sites in nonessential regions of the plasmid to allow 20 insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

[00106] Standard transfection methods may be used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of ENaC protein, which are then purified using standard techniques (see, e.g., Colley *et al.*, *J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of

eukaryotic and prokaryotic cells are performed according to standard techniques (see, e.g., Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983). Alternatively, in a preferred embodiment of the invention, oocytes that 5 express human ENaC subunits are produced by microinjection of cRNA encoding said subunits therein.

[00107] Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, 10 biolistics, liposomes, lipids optimized for DNA transfection, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (see, e.g., Sambrook *et al.*, *supra*). It is only necessary that the particular genetic engineering procedure used be capable 15 of successfully introducing at least one ENaC subunit gene into a host cell, preferably mammalian capable of expressing functional ENaC.

[00108] After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of ENaC subunit(s). In one embodiment, the cells are transiently transfected with all 20 three hENaC genes using lipid-based transfection and cultured for 24 -48 hours prior to performing the screen for ENaC modulators.

[00109] As noted previously, a preferred embodiment of the invention comprises an oocyte expression system. Those methods generally include frog surgery, oocyte isolation, cRNA preparation and oocytes microinjection.

General procedures for frog surgery and oocyte isolation are conventionally known in the art. (See, Marcus-Sekur, et al., *Methods in Enzymol.* 152:284-288 (1987); Goldin, *Methods in Enzymol.* 207:266-279.) Likewise, methods for preparing cRNA are also well known and are reported, e.g., in Swansen, 5 et al., *Meth. Enzymol.* 207:310-319 (1912), Golden, et al., *Meth. Enzymol.* 217:279-297 (1992). The resultant cRNA is then microinjected into frog oocytes by standard methods. (See, Molteni, et al., *Meth. Enzymol.* 254:458-466 (1975); Hitchcock et al., *Meth. Enzymol.* 152:276-284 (1987).)

## ASSAYS FOR MODULATORS OF ENAC PROTEIN

10

### A. Assays

[00110] Modulation of an ENaC protein can be assessed using a variety of assays; preferably cell-based models as described above. Such assays can be used to test for inhibitors and activators of ENaC, which modulate, block, enhance or inhibit salty taste perception.

15

[00111] Preferably, the ENaC will be comprised of three subunits, alpha (or delta), beta and gamma and preferably the human ENaC subunit encoded by the encoded by SEQ ID NO: 1, 2, 3 or 7 or a human ortholog a conservatively modified variant thereof. Alternatively, the ENaC of the assay will be derived from a non-human epithelial cell. Generally, the amino acid sequence identity 20 of each respective subunit will be at least 80%, preferably at least 85%, or 90%, most preferably at least 95%, e.g., 96%, 97%, 98% or 99% to the polypeptide encoded by SEQ ID NO: 1, 2, 3 or 7.

[00112] Measurement of the effect of a candidate comprised of an ENaC protein or cell expressing ENaC protein, either recombinant or naturally

occurring, can be performed using a variety of assays, as described herein. Preferably to identify molecules capable of modulating ENaC, assays are performed to detect the effect of various candidate modulators on ENaC activity in an amphibian oocyte or mammalian cell that expresses a functional 5 ENaC. Preferably, such assays will initially contact ENaC with a known ENaC inhibitor prior to the addition of at least one putative ENaC modulator, e.g., ENaC enhancer. Preferably, the inhibitor will be amiloride or an amiloride derivative such as Phenamil.

[00113] The channel activity of ENaC proteins can be assayed using a 10 variety of assays to measure changes in ion fluxes including patch clamp techniques, measurement of whole cell currents, radiolabeled ion flux assays, and fluorescence assays using voltage-sensitive dyes (see, e.g., Vestergaard-Bogind *et al.*, *J. Membrane Biol.* 88:67-75 (1988); Daniel *et al.*, *J. Pharmacol. Meth.* 25:185-193 (1991); Hoevinsky *et al.*, *J. Membrane Biol.* 137:59-70 15 (1994)) and ion-sensitive dyes. For example, nucleic acids encoding one or more subunits of an ENaC protein or homologue thereof can be injected into *Xenopus* oocytes. Channel activity can then be assessed by measuring changes in membrane current. One means to obtain electrophysiological measurements is by measuring currents using patch clamp techniques, e.g., 20 the "cell-attached" mode, the "inside-out" mode, and the "whole cell" mode (see, e.g., Ackerman *et al.*, *New Engl. J. Med.* 336:1575-1595, 1997). Whole cell currents can be determined using standard methodology such as that described by Hamil *et al.*, *Pflugers. Archiv.* 391:185 (1981).

[00114] Channel activity is also conveniently assessed by measuring changes in intracellular ion levels for example using ion sensitive dyes.

[00115] The activity of ENaC polypeptides can be also assessed using a variety of other assays to determine functional, chemical, and physical effects,

5 e.g., measuring the binding of ENaC polypeptides to other molecules, including peptides, small organic molecules, and lipids; measuring ENaC protein and/or RNA levels, or measuring other aspects of ENaC polypeptides, e.g., transcription levels, or physiological changes that affects ENaC activity. When the functional consequences are determined using intact cells or animals, one

10 can also measure a variety of effects such as changes in cell growth or pH changes or changes in intracellular second messengers such as IP3, cGMP, or cAMP, or components or regulators of the phospholipase C signaling pathway. Such assays can be used to test for both activators and inhibitors.

Modulators thus identified are useful for, e.g., as flavorants in foods,

15 beverages and medicines.

#### Cell-based assays

[00116] In another embodiment, at least one ENaC subunit protein is expressed in a cell, and functional, e.g., physical and chemical or phenotypic,

20 changes are assayed to identify ENaC modulators. Cells expressing ENaC proteins can also be used in binding assays. Any suitable functional effect can be measured, as described herein. For example, changes in membrane potential, changes in intracellular ion levels, and ligand binding are all suitable assays to identify potential modulators using a cell based system. Suitable

25 cells for such cell-based assays include both primary cells, e.g., taste

epithelial cells that expresses an ENaC protein and cultured cell lines such as HEK293T cells that express an ENaC. Another preferred expression system will comprise amphibian oocytes. As noted, these assays will preferably initially contact the ENaC expression cell line, e.g., amphibian oocytes or 5 HEK293 with a known ENaC inhibitor, e.g., amiloride or an amiloride derivative such as Phenamil at a concentration that partially inhibits ENaC function, prior to contacting the cell line with at least one putative ENaC modulator. The ENaC protein can be naturally occurring or recombinant. Also, as described above, fragments of ENaC proteins or chimeras with ion channel 10 activity can be used in cell based assays.

[00117] In yet another embodiment, cellular ENaC polypeptide levels are determined by measuring the level of protein or mRNA. The level of ENaC protein or proteins related to ENaC ion channel activation are measured using immunoassays such as western blotting, ELISA and the like with an antibody 15 that selectively binds to the ENaC polypeptide or a fragment thereof. For measurement of mRNA, amplification, e.g., using PCR, LCR, or hybridization assays, e.g., Northern hybridization, RNase protection, dot blotting, is preferred. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic 20 acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

[00118] Alternatively, ENaC expression can be measured using a reporter gene system. Such a system can be devised using an ENaC protein promoter operably linked to a reporter gene such as chloramphenicol

acetyltransferase, firefly luciferase, bacterial luciferase,  $\beta$ -galactosidase and alkaline phosphatase. Furthermore, the protein of interest can be used as an indirect reporter via attachment to a second reporter such as red or green fluorescent protein (see, e.g., Mistili & Spector, *Nature Biotechnology* 15:961-5 964 (1997)). The reporter construct is typically transfected into a cell. After treatment with a potential modulator, and preferably prior thereto treatment with a known ENaC inhibitor, e.g., Phenamil, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

10 10 [00119] In another embodiment, a functional effect related to signal transduction can be measured. An activated or inhibited ENaC will alter the properties of target enzymes, second messengers, channels, and other effector proteins. Assays for ENaC activity include cells that are loaded with ion or voltage sensitive dyes to report channel activity, e.g., by observing 15 membrane depolarization or sodium influx. Assays for determining activity of such receptors can also use known antagonists for ENaC, such as amiloride or phenamil, as controls to assess activity of tested compounds. In assays for identifying modulatory compounds (e.g., agonists, antagonists), changes in the level of ions in the cytoplasm or membrane potential will be monitored 20 using an ion sensitive or membrane potential fluorescent indicator, respectively. Among the ion-sensitive indicators and voltage probes that may be employed are those disclosed in the Molecular Probes 2002 Catalog: ([www.probes.com](http://www.probes.com)), and specific compounds disclosed infra.

[00120] A preferred assay system will use frog oocytes injected with ENaC cRNAs which are contacted with a test compound and then analyzed by the two-electrode voltage clamp electrophysiological recording technique. (See Stuhmer, *Meth. Enzymol.* 207:319-339 (1992); Wagner et al., *Cellular Physiology and Biochemistry* 10:1-12 (2000)).

**Electrophysiological Assay**

[00121] As noted, a preferred assay for identification of compounds that modulate, i.e., enhance, inhibit or block ENaC comprises an electrophysiological assay that monitors changes in electrical current in cells that express human ENaC subunits that are contacted with at least one putative ENaC modulator (enhancer or inhibitor). These assays may use any cell that expresses a functional ENaC. In the preferred embodiment, the cells will comprise oocytes, preferably frog oocytes, mammalian cells, yeast cells or insect cells, or another expression system that is suitable for expressing a functional ENaC ion channel. Preferably, the expression system will exhibit robust and rapid human ENaC sodium channel expression and desirably will not express any or very few endogenous ion channels, thereby facilitating the identification of compounds that specifically modulate ENaC sodium channel function. Thereby, an undesirable background response is minimized or eliminated. Moreover, robust cells, such as oocytes, are desirable as this enables the cells to be reused in assays according to the invention. Oocytes have been reported previously to rapidly and robustly express other functional ion channels including ENaCs (Pascal, *CRC Crit. Rev. Biotech.* 22(4):317-87 (1987); Wagner et al., *Cell Physiol. Biochem.* 10:1-12 (2000); Canessa et al., *Nature* 367:463-467 (1994)).

[00122] A particularly preferred electrophysiological assay is a moderate throughput assay that measures ENaC sodium channel function in frog oocytes by the two-electrode voltage clamp technique. This robust, fast expression system provides for the expression of ~ 1 million ENaC channels

5 in an oocyte membrane after only about 18-24 hours. Moreover, because oocytes are relatively large (1mm in diameter, relatively large compared to most mammalian cells), they are easy to handle and work with.

[00123] Based on these advantages, a single oocyte can be used to obtain multiple and repetitive electrophysiological recording. Also, an oocyte typically

10 expresses few endogenous channels, and expression is at levels below that which cause high background relative to the background seen in some other expression systems, e.g., HEK293T cells. Thereby, oocytes allow for repeated direct measurement of the effect of target compounds on ENaC sodium channel function.

15 [00124] In a preferred two-electrode voltage clamp assay according to the invention (exemplified in detail in the Example 4 *infra*), frog oocytes that have been microinjected with ENaC  $\alpha$ ,  $\beta$ , and  $\gamma$  human ENaC cRNAs (or  $\delta$ ,  $\beta$  and  $\gamma$  human ENaC cRNAs) are transferred to glass scintillation vials and incubated under appropriate conditions to facilitate ENaC protein expression.

20 [00125] After ENaC sodium ion channel expression is obtained, typically around 24 hours post-cRNA microinjection, ENaC function is measured according to the two-electrode voltage clamp technique using an appropriate two-electrode voltage measuring device, e.g., OpusXpress 6000A parallel

oocyte voltage clamp system (Axon Instruments). The two-electrode voltage clamp technique measures the macroscopic electrical current flowing across the entire oocyte membrane through the ENaC sodium ion channels. Oocytes are punctured with a voltage-sensing electrode and a current sensing 5 electrode; the voltage, or potential difference across the oocyte membrane, is clamped to a particular value using the voltage-sensing electrode and the current, or the flow of ions across the oocyte membrane, required to maintain the voltage is measured using the current-sensing electrode. The OpusXpresss system is one example of a commerically available two-10 electrode voltage measuring device which is semi-automated and which comprises a workstation that permits electrophysiological recordings to be made from 8 oocytes simultaneously. This system also provides for automated oocyte impalement and delivery of target compounds by a computer-controlled fluid handler that delivers compound into 96-well 15 compound plates. This system can best be described as a medium or moderate-throughput system as it allows for the evaluation of about 60 compounds per week. Of course more compounds can be screened by the addition of other voltage measuring devices, as described.

[00126] In this assay system, ENaC enhancers will result in an increase in 20 current passing through the ENaC channels in the oocyte membrane. This value is calculated by a standard formula provided *infra* in (Example 4). Such assays also may include appropriate negative controls, e.g., amiloride, which is a known ENaC inhibitor that blocks sodium transport through ENaC channels. Therefore, this compound functions both as an internal control to 25 verify that oocytes express functional ENaC, and, in oocytes exhibiting

amiloride inhibition, allows for the screening of putative ENaC enhancers after amiloride compound is applied (if the target compound is an ENaC enhancer it will result in an increase in current passing through ENaC channels in the oocyte membrane).

5 [00127] Desirably, a % enhancement factor is calculated for each enhancer. For example, a 100% enhancer increases ENaC activity 100% relative to the basal control value (no compound).

[00128] Negative controls are also desirably performed to confirm that oocytes which are not injected with ENaC cRNAs do not exhibit the same

10 effects.

[00129] As discussed in greater detail in Example 4 *infra*, more complex analyses are also desirably performed on compounds that exhibit maximal % enhancement values e.g., current/voltage (I/V) curves, amiloride competitive experiments and dose-response curves to determine the concentration at 15 which the compound exhibits half-maximal activity (EC50 value). These experiments will further confirm that the effect of the compound is ENaC-specific.

[00130] These assays will provide for the identification of ENaC modulators, preferably ENaC enhancers, which may be used as additives for foods,

20 beverages, pharmaceuticals and the like in order to modulate the salty taste associated therewith. Desirably, an ENaC enhancer will exhibit at least 20% enhancement factor, more preferably at least 50% and even more preferably at least an 100% enhancement factor. These oocyte-based assays are

discussed in further detail as well as the intrinsic advantages associated therewith in Example 4 of this application.

**Animal models**

[00131] Animal models that express hENaC also find use in screening for 5 modulators of salty taste. Similarly, transgenic animal technology including gene knockout technology, for example as a result of homologous recombination with an appropriate gene targeting vector, or gene overexpression, will result in the absence or increased expression of the ENaC protein. The same technology can also be applied to make knockout 10 cells. When desired, tissue-specific expression or knockout of the ENaC protein may be necessary. Transgenic animals generated by such methods find use as animal models of responses to salty taste stimuli.

[00132] Knockout cells and transgenic mice can be made by insertion of a 15 marker gene or other heterologous gene into an endogenous ENaC gene site in the mouse genome via homologous recombination. Such mice can also be made by substituting an endogenous ENaC with a mutated version of the ENaC gene, or by mutating an endogenous gene.

[00133] A DNA construct is introduced into the nuclei of embryonic stem 20 cells. Cells containing the newly engineered genetic lesion are injected into a host mouse embryo, which is re-implanted into a recipient female. Some of these embryos develop into chimeric mice that possess germ cells partially derived from the mutant cell line. Therefore, by breeding the chimeric mice it is possible to obtain a new line of mice containing the introduced genetic lesion (see, e.g., Capecchi *et al.*, *Science* 244:1288 (1989)). Chimeric

targeted mice can be derived according to Hogan *et al.*, *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988) and *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, ed., IRL Press, Washington, D.C., (1987).

5

#### B. Modulators

[00134] The compounds tested as modulators of ENaC protein can be any small organic molecule, or a biological entity, such as a protein, e.g., an antibody or peptide, a sugar, a nucleic acid, e.g., an antisense oligonucleotide

10 or a ribozyme, or a lipid. Alternatively, modulators can be genetically altered versions of an ENaC protein. Typically, test compounds will be small organic molecules, peptides, lipids, and lipid analogs. Preferably, the tested compounds are safe for human consumption.

[00135] Essentially any chemical compound can be used as a potential

15 modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in  
20 microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including ChemDiv (San Diego, CA), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica-Analytika (Buchs Switzerland) and the like.

[00136] In the preferred embodiment, moderate or high throughput screening methods involve providing a small organic molecule or peptide library containing a significant number of potential ENaC modulators (potential activator or inhibitor compounds). Such "chemical libraries" are then 5 screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual products. As noted, the preferred oocyte two-voltage clamp electrode 10 system (a single device) permits about 60 compounds to be tested per week.

[00137] A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library 15 is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

[00138] Preparation and screening of combinatorial chemical libraries is well 20 known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not

limited to: peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and 5 dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 10 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (see Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (see, e.g., U.S. Patent 5,539,083), antibody libraries (see, e.g., Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and 15 PCT/US96/10287), carbohydrate libraries (see, e.g., Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (see, e.g., benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; 20 morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

[00139] Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, 25 Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous

combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, RU, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

5

**FOODS AND BEVERAGE COMPOSITIONS CONTAINING ENaC MODULATORY COMPOUND IDENTIFIED USING DISCLOSED ASSAYS**

[00140] The compounds identified using the disclosed assays, e.g., the electrophysiological (two electrode voltage-clamp technique) assays and fluorescence cell-based assay disclosed in the examples, are potentially

10 useful as ingredients or flavorants in ingestible compositions, i.e., foods and beverages as well as orally administered medicinals. Compounds that modulate or enhance salty taste perception can be used alone or in combination as flavorants in foods or beverages. In the preferred application, the modulator will be incorporated into a food or beverage with a reduced  
15 level of sodium and the salty taste of the resulting product will be similar to that of the high sodium product. Examples of such foods and beverages include snack foods such as pretzels, potato chips, crackers, soups, dips, soft drinks, packaged meat products, among others.

[00141] Alternatively, compounds that block or inhibit salty taste perception  
20 can be used as ingredients or flavorants in foods that naturally contain high salt concentrations in order to block or camouflage the salty taste thereof.

[00142] The amount of such compound(s) will be an amount that yields the desired degree of salty taste perception. Of course compounds used in such

applications will be determined to be safe for human consumption and to be acceptable in human taste tests.

**PREFERRED ASSAY EMBODIMENT USING PHENAMIL OR EQUIVALENT**

5 [00143] As disclosed *supra*, one of the preferred embodiments of the invention will comprise contacting a test cell expressing a functional ENaC with at least one putative modulator compound in the presence of a membrane potential dye, and preferably prior thereto contacting said test cell with at least one compound known to modulate (inhibit) ENaC function,

10 preferably an amiloride derivative such as Phenamil and monitoring the activity of the ENaC expressed by the test cell to determine the extent of ENaC modulation. As noted, the addition of an ENaC inhibitor prior to the test compound improves assay results. This inhibitor, e.g., Phenamil, is used at a concentration that at least partially inhibits ENaC function. The method can

15 further comprise evaluating the putative modulator compound for *in vivo* effects on salty taste perception (e.g., performing tasting experiments to determine the *in vivo* effect on salty taste perception). For example, cDNAs encoding the ENaC subunits are cloned from human kidney cell cDNA, human lung cell cDNA, or human taste cell cDNA. As mentioned above,

20 native ENaC is a multimeric protein consisting of three subunits (alpha or delta, beta, and gamma). ENaC functions as a constitutively active  $\text{Na}^+$  selective cation channel, is found in taste buds as well as other tissues, and is a candidate human salt receptor underlying the physiological perception of salty taste.

[00144] In another preferred embodiment of the invention, such a method is carried out in a high throughput assay format using multi-well plates and a fluorescence intensity plate reader (e.g., Aurora Biosciences VIPR instrument or Molecular Device's FLIPR instrument). The test cells may be seeded, dye-loaded, optionally, preferably initially contacting the test cells with a known ENaC inhibitor at a concentration whereby ENaC function is at least partially and preferably reversibly inhibited, thereafter contacting said test cell with at least one test compound, and monitoring fluorescence intensity in the same multi-well plate. Such an assay format can reliably detect both activation or inhibition of ENaC function, providing a robust screen for compounds that could either enhance or block channel activity. The assay described above has been optimized to identify ENaC enhancers. The assay described herein thus has advantages over existing assays, such as those described above, in that a human ENaC is utilized, mammalian cells are employed and the assay can be run in standard multi-well (e.g., 96, 384, or 1536 well) plates in high-throughput mode. (However, as discussed above, mammalian cells possess some disadvantageous properties, e.g., they may express endogenous ion channels at levels resulting in undesirable background levels.)

[00145] In this preferred embodiment of the invention, cells, preferably mammalian cells, will be produced that functionally express at least the alpha (or delta) subunit of ENaC. In preferred embodiments, all three subunits of hENaC ( $\alpha$  or  $\delta$ ,  $\beta$ , and  $\gamma$ ) are expressed either transiently or stably. The ENaC subunit(s) employed can be naturally occurring forms, variants containing SNPs, alternatively spliced forms, combinations of forms or any functional variants known in the art (see e.g., accession numbers P37088, P51168,

P51170, and P51172). Preferably, the ENaC will be comprised of the human alpha, beta and gamma ENaC subunits encoded by the nucleic acid sequence in SEQ ID NO. 1, 2, 3 or the human beta, gamma and delta ENaC subunits encoded by SEQ ID NO. 2, 3 and 7. The mammalian cells can be 5 any type known in the art such as COS, CHO, BHK, MDCK, HEK293, or HEK293T (human embryonic kidney cells expressing the large T-cell antigen). Preferably, the cell is HEK293T. The cells can be transfected using standard methods known in the art, such as but not limited to  $\text{Ca}^{2+}$  phosphate or lipid-based systems, or methods previously mentioned.

10 [00146] These transfected cells are then preferably seeded into multi-well culture plates. Functional expression is then allowed to proceed for a time sufficient to reach at least about 70% confluence, more preferably to at least about 80% confluence or to form a cell layer dense enough to withstand possible fluid perturbations caused by compound addition. Generally, an 15 incubation time of at least 24 hours will be sufficient, but can be longer as well. The cells are then washed to remove growth media and incubated with a membrane-potential dye for a time sufficient to allow the dye to equilibrate across the plasma membranes of the seeded cells. One of skill in the art will recognize that the dye loading conditions are dependent on factors such as 20 cell type, dye type, incubation parameters, etc. In one embodiment, the dye may be used at about 2  $\mu\text{M}$  to about 5  $\mu\text{M}$  of the final concentration. Further, the optimal dye loading time may range from about 30 to about 60 minutes at 37  $^{\circ}\text{C}$  for most cells. In the preferred embodiment, the membrane potential dyes are from Molecular Devices (cat# R8034). In other embodiments,

suitable dyes include e.g., single wavelength-based dyes such as DiBAC, DiSBAC (Molecular Devices), and Di-4-ANEPPS (Biotium), or dual wavelength FRET-based dyes such as DiSBAC2, DiSBAC3, and CC-2-DMPE (Aurora Biosciences). [Chemical Names - Di-4-ANEPPS (Pyridinium, 4-(6-5 (dibutylamino)-2-naphthalenyl)ethenyl) -1-(3-sulfopropyl)-, hydroxide, inner salt), DiSBAC4(2) (bis-(1,2-dibarbituric acid)-trimethine oxanol), DiSBAC4(3) (bis-(1,3-dibarbituric acid)-trimethine oxanol), CC-2-DMPE (Pacific Blue™ 1,2-ditetradecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt) and SBFI-AM (1,3-Benzenedicarboxylic acid, 4,4'-[1,4,10-trioxa-7,13-dia-10 zacyclopentadecane- 7,13-diylbis(5-methoxy-6,12-benzofurandiyl)] bis-, tetra-kis[(acetyloxy)methyl] ester].

[00147] In one embodiment, the dye-loaded cells are preferably contacted with a known ENaC inhibitor, e.g., Phenamil, then contacted with test compounds (or controls), and the cell cultures are monitored using standard 15 fluorescence analysis instrumentation such as or VIPR or FLIPR®. The addition of NaCl or other test compounds which pharmacologically act on ENaC elicit a change in membrane potential which is then detected as a change in the resting fluorescence in a standard fluorescence intensity plate reader (e.g., FLIPR) or voltage intensity plate reader (e.g. VIPR). As such, the 20 method of the present invention can be used to identify salty taste modulating compounds by monitoring the activity of ENaC in the test cells through fluorescence. For instance, a decrease in fluorescence may indicate a taste (salty) blocker, while an increase in fluorescence may indicate a taste (salty) enhancer.

[00148] Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting. It is understood that various modifications and changes can be made to the herein disclosed 5 exemplary embodiments without departing from the spirit and scope of the invention.

#### EXAMPLE 1

[00149] DNA sequences encoding the alpha, beta and gamma subunit of a human ENaC expressed in human taste cells were cloned from human kidney 10 cells by RT-PCR.

#### Methods for cloning human epithelium sodium channel subunit DNA sequences (ENaCs)

[00150] Human ENaC cDNAs for  $\alpha$ ,  $\beta$  and  $\gamma$  ENaC were amplified from human kidney cDNA (Origene Technologies Inc.) by PCR using the following 15 primer pairs, respectively: 5' CGC GGA TCC GCC CAT ACC AGG TCT CAT G 3' and 5' CCG GAA TTC CTG CAC ATC CTT CAA TCT TGC 3'; 5' CGC GGA TCC AGC AGG TGC CAC TAT GCA C 3' and CCG CTC GAG GTC TTG GCT GCT CAG TGA G 3'; 5' CGC GGA TCC CCT CAA AGT CCC ATC CTC G 3' and 5' CCG GAA TTC GAC TAG ATC TGT CTT CTC AAC 3'. The primers 20 were designed to be complementary to 5' and 3'-untranslated region sequence in order to retain the endogenous translation initiation signal, and they introduced terminal restriction endonuclease sites that were used to clone amplified ENaC cDNAs into the mammalian expression vector pcDNA3 (Invitrogen) for functional expression experiments. The cloned ENaC cDNAs 25 were sequenced and compared to ENaC sequences in public DNA databanks.

Each cloned subunit is a composite of polymorphisms present in different databank alleles; that is, every polymorphism in each cloned subunit identified by pairwise comparison of the cloned subunit to a databank allele could be found in another databank allele. In addition, polymorphisms in cloned ENaC 5 subunits were verified by sequencing of cloned cDNAs amplified in independent PCR experiments.

[00151] The nucleic acid sequences encoding cloned sequences alpha, beta and gamma hENaC subunits are respectively contained in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3 and the corresponding amino acid 10 sequences in SEQ ID NO: 4, 5 and 6. Each of these DNA sequences was inserted into the expression vector pcDNA3 to produce alpha, beta and gamma subunit plasmids that express human ENaC subunit polypeptides. Also, the nucleic acid sequence for the human amiloride sensitive sodium channel delta subunit ( $\Delta$ NaCh) is contained in SEQ ID NO: 7, which functions 15 equivalently to the ENaC alpha subunit. The amino acid sequence for the delta subunit is contained in SEQ ID NO: 8. HEK293T cells were transiently transfected via  $\text{Ca}^{2+}$  phosphate with 1:1:1 weight ratios of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunit plasmids expressing human ENaC. Such transfection resulted in a  $\text{Na}^+$  dependent amiloride sensitive fluorescence change, as compared to mock- 20 transfected cells. With reference to Figure 1, samples A, B, C, and D were transfected with 1:1:1: ratios of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunit plasmids at absolute levels of 4, 4, 1, and 0.25 micrograms, respectively. Samples E and F were mock transfected with Beta-gal and pUC DNAs. Transfection efficiency was approximately 40% and cell density was approximated 70%. Cells were

analyzed using a FLIPR I (Molecular Devices) instrument using a membrane-potential fluorescent dye. All traces shown are from a single plate with A (n=4), B, C, D, E, (n=12), and F (n=8).

[00152] As depicted in Figures 1, 2, and 3, sodium-dependant amiloride-sensitive changes in resting potential (hENaC responses) were not significantly affected in untransfected HEK293T cells. Further, such resting potential changes were greatly enhanced in cells transfected with all three subunits of the hENaC compared to cells tranfected with only the alpha subunit of hENaC (data not shown). Moreover, the ability of NaCl to induce membrane potential changes, and the effect of amiloride to block hENaC channel activity follow dose response-relationships similar to that reported in the literature using low throughput electrophysiological recording.

## EXAMPLE 2

[00153] DNA sequences encoding the alpha, beta and gamma subunits of a human ENaC, SEQID 1, 2, and 3, respectively, were each cloned into the expression vector pcDNA3 to produce alpha, beta and gamma subunit plasmids that express human ENaC subunit polypeptides. HEK293T cells were transiently transfected via lipofection with 1:1:1 weight ratios of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunit plasmids expressing human ENaC (2 $\mu$ g of each subunit/20 million cells). Transfected cells were plated into 384-well plates and analyzed on a VIPRII Instrument (Aurora Biosciences) using voltage-sensitive fluorescent dyes. Cells expressing ENaC exhibited a  $\text{Na}^+$  dependent fluorescence change, as compared to mock-transfected cells (Figure 1). In figure 2, the  $\text{Na}^+$ -dependent fluorescence change is totally abolished by Phenamil, a

known ENaC antagonist. Conversely, another compound was found to increase the Na<sup>+</sup>-dependent fluorescence change but this effect is abolished by Phenamil. This compound is believed to be an ENaC enhancer, as it acts opposite to Phenamil in this assay for ENaC function.

5 [00154] Methods and Materials for Example 2:

1. All materials used are identified below in the "Materials Section".
2. HEK293T cells are grown to 80% confluence and dissociated from the culture dishes with an enzymatic solution (Trypsin/EDTA) for 3 minutes at 37C. Detached cells are analyzed for density and viability using a bench top flow cytometer (Guava; Guava Technologies). Cells with less than 85% viability are discarded from the experiment.

10 [The procedures herein are conditions for transfection of HEK293T cells equivalent to ten screening 384-well plates (200,000,000 cells). These conditions can be altered e.g., by increasing or decreasing cell confluency by use of different size multi-well plates etc.]

15

3. Dissociated cells are washed and recovered in their culture medium (complete) at a density of ~1,000,000 cells/ml. Mammalian expression plasmid DNAs encoding the human ENaC subunits are mixed in an eppendorf in an equal ratio (10ug  $\alpha$ ; 10ug  $\beta$  and 10ug  $\gamma$ /20,000,000 cells). 170 $\mu$ g of carrier plasmid DNA (pUC-18) is then added to the DNA mix (for a total of 200 $\mu$ g DNA/200,000,000 cells). 557 $\mu$ l of the transfection reagent TransIT (Panvera Corporation) is added to 20 ml of culture medium exempt of serum and antibiotic. The DNA solution is then added to the TransIT solution and the DNA-lipid solution is incubated at room temperature. After 60 minutes, the DNA-lipid complexes are transferred into the cell solution and volume is adjusted to 320 ml with complete cell culture medium for a final density of

635,000 viable cells/ml. (As discussed previously, the alpha subunit DNA may be interchanged with the delta subunit DNA and used to produce recombinant cells that express a functional ENaC comprised of the beta, gamma and delta ENaC subunits.)

5

4. Black 384-well poly-D-lysine clear bottom screening plates (Becton Dickinson) are coated with 40 $\mu$ l/well of a Matrigel solution (20 $\mu$ g/ml; Collaborative Biomedical Products) for 1 hour at room temperature.

10

Coating solution is removed and plates are kept at room temperature until cell plating.

5. The cell/DNA solution is plated with a Multidrop into 384 well plates at a density of 50,000 cells/well (80 $\mu$ l/well).

15

6. 27 hours after plating, cells are washed and loaded with the membrane potential sensitive dyes (CC2-DMPE and DiSBAC2(3)) as described below.

20

7. Cells are stimulated with 200 $\mu$ M compounds ([2x]) and read on line using a Voltage Intensity Plate Reader (VIPRII; Aurora Biosciences Corporation). Other concentrations of compounds can be used in the assay. Buffer preparation and plate layout are described below in the VIPR. The assay is performed at "room temperature", typically about 22°C, but can also be performed at other temperatures by preheating or cooling the cells and reagents prior to addition of compounds.

25

### Materials

1. HEK 293T cells growing on 150 cm<sup>2</sup> flask (Becton Dickinson 0.2um vented Blueplug seal cap) (37°C, 6% CO<sub>2</sub>)

30

2. Dulbecco's Modified Eagle Medium (DMEM) (cat #11965-092 Gibco BRL) (Kept at 4°C)

3. DMEM with HEPES (DMEMH) (cat #12430-054, Gibco BRL) (Kept at 4°C)

4. Foetal Bovine serum (FBS) (cat#10082-147, Gibco BRL) (Kept in – 20°C)

5. Trypsin EDTA (1 x) (cat#25200-072 Gibco-BRL) (Kept in –20°C)

6. Transit-293 (cat#MIR2705, Panvera) (Kept in 4°C)

5 7.  $\alpha$ ,  $\beta$ , and  $\gamma$  ENaC DNA preparations (1  $\mu$ g/ $\mu$ L each) (Kept in 4°C)

8. pUC18 carrier DNA ((1  $\mu$ g/ $\mu$ L) (Kept in 4°C)

9. Matrigel (cat #40230, Collaborative Biomedical Products)

2. Cell loading

10

HBSS – Hank's Buffered Saline Solution

DiSBAC<sub>2</sub>(3) 5 mM in 100% DMSO 2.5 $\mu$ M

ESS-CY4 or VABSC-1 200 mM in dH<sub>2</sub>O 350 $\mu$ M

VIPR NMDG BUFFER – see formula in "VIPR Plate Layout" section

15 below:

Components	To Make Volume				ml
	10 ml	50 ml	100	200	
CC2-DMPE ( $\mu$ )	20	100	200	400	
Pluronic ( $\mu$ )	20	100	200	400	
HBSS (ml)	10	50	100	200	
DiSBAC <sub>2</sub> (3) ( $\mu$ )	5	25	50	100	
ESS ( $\mu$ )	17.5	87.5	175	350	
VIPR NMDG Buffer (ml)	10	50	100	200	

Preparation of CC2-DMPE Loading Buffer

1. Mix equal volumes of the CC2-DMPE stock solution and Pluronic F127.

20 2. Add the CC2-DMPE/Pluronic mix to HBSS while vortexing.

Loading of cells with CC2-DMPE

1. Remove cells from CO<sub>2</sub> incubator.
2. Look for variation of density/well
3. Prime EMLA with HBSS
- 5 4. Wash cells with HBSS 3X80ul to remove residual growth medium and serum
5. Add 40  $\mu$ L of 10 $\mu$ M CC2-DMPE loading buffer to each well
6. Look for variation of density/well
7. Incubate for 30 minutes at room temperature in the dark.

10

Preparation of DiSBAC<sub>2</sub>(3) loading buffer

(can be done during CC2 incubation)

- 15 1. Mix DiSBAC<sub>2</sub>(3) and ESS-CY4 or VABSC-1, plus double volume of PluronicF127 of DiSBAC2(3)
2. Add the above mix to VIPR NMDG BUFFER, vortex

Loading of cells with DiSBAC2(3) loading buffer

- 20 1. Prime EMLA with NMDG buffer
2. Wash CC2-DMPE-loaded cells using VIPR NMDG buffer as the wash buffer, 3X80 $\mu$ /well
3. Add 40 $\mu$  of 2.5 $\mu$ M DiSBAC2(3), 350 $\mu$ M ESS-CY4 or VABSC-1 loading buffer to each well
- 25 4. Look for variation of density/well
5. Incubate for 20 minutes at room temperature in the dark before running on VIPR II

VIPR Plate Layout

ENaC VIPR compound plate preparation 384 well format										NMDG Buffer:	NaCl Buffer:	High K Buffer:	"VIPR NONE BUFFER"					
Prepare enough of the following buffers to load all plates:										130mM NMDG	150mM NaCl							
Stock Solutions:			Final							2mM KCL	2mM KCL	160mM KCl	2mMKCl					
Pluronic F127	100 mg/ml in 100% DMSO									2mM CaCl2	2mM CaCl2	2mMCaCl2*2H2O						
DISBAC2(3)	5 mM in 100% DMSO		2.5µM							1mM MgCl2	1mM MgCl2	1mMMgCl2*6H2O						
ESS-CY4 or VABSC-1	200 mM in dH <sub>2</sub> O		350µM							10mM Hepes	10mM Hepes	10mM Hepes 10mMHEPES acid						
VIPR NMDG BUFFER										5mM D-glucose	5mM D-glucose	5mM D-glucose 5mM D-glucose						
VIPR Na <sup>+</sup> BUFFER													pH 7.3 w/ Trisbase					
VIPR High K buffer																		
Final volume	10mls	15mls	20mls	100mls	200mls	400mls	500mls											
DISBAC2(3) (µl)	5	7.5	10	50	100	200	250											
ESS or VABSC-1 (µl)	17.5	26.25	35	175	350	700	875											
Pluronic F127	10	15	20	100	200	400	500											
Preparation of Column 1 top half Buffer																		
1. Mix DISBAC2(3) and ESS-CY4 or VABSC-1 and Pluronic F127 into VIPR NMDG buffer while vortex																		
Preparation of Column 1 bottom half Buffer																		
1. Mix DISBAC2(3) and ESS-CY4 or VABSC-1 and Pluronic F127 into VIPR HighK buffer while vortex																		
Preparation of Column 24 buffer																		
Add VIPR NMDG buffer into Column 24																		
Preparation of Column 2 23 70mM NaCl loading buffer										70mM NaCl	NaCl Buffer:	NMDG Buffer:						
1. Mix VIPR Na <sup>+</sup> buffer with VIPR NMDG buffer to make 70mM NaCl										30mls	14	16						
2. Mix DISBAC2(3) and ESS-CY4 or VABSC-1 and Pluronic F127 into the above buffer while vortex 300mls										400mls	140	160						
											214.6	245.3						
Adding 2µl of 100% DMSO into Column1, 2 and 24																		
Adding 2µl of 10mM compound X in 100% DMSO to Column23																		
Adding all the other compound into Column3 22.																		
Plate layout	1	2	3	4	5	6	7	8	9	10	11,12	13,14	15,16	17,18	19,20	21,22	23	24
A	NMDG 70mMNaCl															70mM Na+200µM X	NMDG	
B	NMDG 70mMNaCl															70mM Na+200µM X	NMDG	
C	NMDG 70mMNaCl															70mM Na+200µM X	NMDG	
D	NMDG 70mMNaCl															70mM Na+200µM X	NMDG	
E	NMDG 70mMNaCl															70mM Na+200µM X	NMDG	
F	NMDG 70mMNaCl															70mM Na+200µM X	NMDG	
G	NMDG 70mMNaCl															70mM Na+200µM X	NMDG	
H	NMDG 70mMNaCl															70mM Na+200µM X	NMDG	
I	HighK 70mMNaCl															70mM Na+200µM X	NMDG	
J	HighK 70mMNaCl															70mM Na+200µM X	NMDG	
K	HighK 70mMNaCl															70mM Na+200µM X	NMDG	
L	HighK 70mMNaCl															70mM Na+200µM X	NMDG	
M	HighK 70mMNaCl															70mM Na+200µM X	NMDG	
N	HighK 70mMNaCl															70mM Na+200µM X	NMDG	
O	HighK 70mMNaCl															70mM Na+200µM X	NMDG	
P	HighK 70mMNaCl															70mM Na+200µM X	NMDG	

**EXAMPLE 3**  
**Improved ENaC Assay Using ENaC Inhibitor (Phenamil)**

[00155] Modulation of ENaC functions is monitored in human embryonic kidney (HEK)293 cells expressing the three different ENaC subunits. HEK293 5 cells are transiently transfected with the ENaC subunit plasmids using lipid-based systems. Transfected HEK293 cells are seeded into 384-well screening plates and functional expression of ENaC is allowed to proceed for a total of 24 hours. Cells are then labeled with specific dyes (such as DisBac2(3) and CC2-DMPE, Panvera) allowing the detection in subtle 10 changes in membrane potential. Changes in dye fluorescence properties, upon modulation of ENaC functions, are monitored using a Voltage-Intensity-Plate-Reader (VIPR, Panvera). Using this technology, we can detect inhibition of ENaC function (Na<sup>+</sup>-induced change in membrane potential dye fluorescence) with increasing concentration of a known ENaC inhibitor, 15 Phenamil (Figure 6, blue trace). On the other hand an ENaC enhancer (ID # 478354) increases ENaC activity by roughly 18% (Figure 6, difference between red and blue data points in the black rectangle). Notably, the effect of compound 478354 is greatly improved by increasing concentrations of Phenamil (Figure 6, difference between the red and blue data points in yellow 20 rectangle). Under these conditions, 478354 increases ENaC activity by as much as 50-60% in the presence of 0.2 to 0.5 $\mu$ M Phenamil. We conducted more than 241 and 835 independent experiments in the absence and 25 presence of 0.5  $\mu$ M Phenamil respectively (Figure 7A and 7B). During these experiments, we determined the effect of 478354 on the assay window using a Z' factor. Z' is a statistical parameter used to judge the quality of a signal window by quantifying the separation of high and low controls sets with

respect to their variance. A  $Z' \geq 0$  and  $\leq 1$  indicates a meaningful signal window by which tested chemistries can be compared during screening. In the absence of Phenamil, a signal window determined by the 478354 high control standard failed in more than 90% of experiments (Figure 2A). However, in the 5 presence of Phenamil, 478354 significantly increased the signal window in 85% of experiments, with most of the  $Z'$  centered around 0.26 (Figure 2B).

[00156] These results indicate that the use of an ENaC inhibitor such as Phenamil, prior to screening with one or more potential ENaC modulators, e.g., ENaC enhancers, enhances signal intensity, thereby significantly 10 improving the likelihood of identifying molecules enhancing ENaC activity in throughput cell-based assays.

#### EXAMPLE 4

##### Electrophysiological Assay For Identifying ENaC Modulators Using Amphibian Oocytes That Express Functional Human ENaC

15 [00157] The oocyte expression system has intrinsic advantages (expression levels, robust, low endogenous ion channel expression, et al.) that render it useful to examine the effects of compounds on sodium transport through ENaC channels. These compounds are candidates for enhancing salt taste perception. The oocyte expression system has been used earlier for the rapid 20 and robust expression of ion channels, including ENaC, in functional studies (Dascal, CRC Crit. Rev. Biochem. (1987) 22(4): 317-387; Wagner, et al, Cellular Physiology and Biochemistry (2000) 10: 1-12; Canessa, et al, Nature (1994) 367: 463-467). Therefore, this system was selected for use in a two-electrode voltage clamp assay using methods and materials as described 25 below.

[00158] The oocyte expression system is comprised of the following steps and methodologies, which collectively comprise the screen for ENaC enhancers: frog surgery and oocyte isolation, cRNA preparation, oocyte microinjection, and measurement of ENaC currents in oocytes using two-electrode voltage clamp electrophysiological recordings. The following references describe general practices for frog surgery and oocyte isolation (Marcus-Sekura, et al, Methods in Enzymology (1987) 152: 284-288; Goldin, Methods in Enzymology (1992) 207: 266-279), cRNA preparation (Swanson, et al, Methods in Enzymology (1992) 207: 310-319; Goldin, et al, Methods in Enzymology (1992) 207: 279-297), oocyte microinjection (Matten, et al, Methods in Enzymology (1995) 254: 458-466; Hitchcock, et al, Methods in Enzymology (1987) 152: 276-284), and two-electrode voltage clamp electrophysiological recording (Stuhmer, Methods in Enzymology (1992) 207: 319-339; Wagner, et al, Cellular Physiology and Biochemistry (2000) 10: 1-12). Each of these methodologies, as they pertain to the screen for ENaC enhancers, is described in further detail below.

### Frog Surgery and Oocyte Isolation

[00159] Female *Xenopus laevis* South African clawed frogs greater than or equal to 9 cm in length are obtained from NASCO (Fort Atkinson, WI). Frogs are anesthetized in 0.15% MS-222 (tricaine or ethyl-3-aminobenzoate methanesulfonate; Sigma) in distilled water and placed on ice. Using sterile surgical tools, sequential 1-2 cm incisions are made in the abdomen through both the outer skin layer and the inner peritoneal layer. Excised ovarian lobes

(containing 1000-2000 oocytes) are placed in OR-2 calcium-free media (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES pH 7.5 with NaOH) and sequentially digested with 2 mg/ml collagenase type IA (Sigma), prepared immediately before use, for 45 min followed by 1 mg/ml collagenase type IA

5 for 15 min on a rocking platform at room temperature. After enzymatic digestion, at which point the majority of oocytes are released from the ovarian lobes, oocytes are rinsed in OR-2 without collagenase and transferred to a Petri dish containing Barth's saline (88 mM NaCl, 2 mM KCl, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, 2.4 mM NaHCO<sub>3</sub>, and 5 mM HEPES 10 pH7.5; Specialty Media) supplemented with 2.5 mM sodium pyruvate. Mature stage V or VI oocytes (~1 mm diameter) containing distinct animal poles, corresponding to the dark side of the egg containing melanin pigment granules, and vegetal poles, corresponding to the light side of the egg containing yolk proteins, are selected for microinjection. Frogs are sutured 15 using a C6 needle with a 3-0 black braid suture (Harvard Apparatus) and resused for oocyte isolation following a 2-3 month recovery period.

### cRNA Preparation

[00160] ENaC cRNA is generated using the mMESSAGE mMACHINE kit

20 according to the manufacturer's instructions (Ambion) from  $\alpha$ ,  $\beta$ , and  $\gamma$  human ENaC DNA plasmids described in our previous patent (PCT WO 02/087306 A2) using T7 RNA polymerase to transcribe cRNA in vitro from DNA linearized with Eco RI for  $\alpha$  and  $\gamma$  ENaC and linearized with Xho I for  $\beta$  ENaC. cRNA quality is checked by denaturing agarose gel electrophoresis and

spectrophometric absorbance readings at 260 and 280 nm to ensure that full-length, non-degraded cRNA is generated.

**Microinjection**

[00161] Microinjection needles are pulled on a Model P-97 Flaming/Brown

5 Micropipette Puller (Sutter Instrument Co.) using borosilicate glass capillaries (World Precision Instruments Inc.), back-filled with mineral oil (Sigma), and then front-filled with ENaC cRNA using a Nanoliter 2000 injector with a Micro4 MicroSyringe Pump Controller (World Precision Instruments). Oocytes are microinjected in the animal pole with 10-15 nl containing 1-3 ng of each  $\alpha$ ,  $\beta$ ,  
10 and  $\gamma$  human ENaC cRNA. Following microinjection, oocytes are transferred to glass scintillation vials containing Barth's solution supplemented with 2.5 mM sodium pyruvate and incubated at 18-19 °C overnight under normal atmospheric conditions. During this time, the oocytes translate injected ENaC cRNA into protein.

15 **Measurement of ENaC Currents in Oocytes using Two-Electrode Voltage Clamp Electrophysiological Recordings**

[00162] Twenty-four hours post-microinjection, ENaC function is measured in oocytes using the two-electrode voltage clamp technique on an OpusXpress 6000A parallel oocyte voltage clamp system (Axon Instruments).

20 The two-electrode voltage clamp technique is an electrophysiology method that measures the macroscopic electrical current flowing across the entire oocyte membrane through protein channels such as ENaC (Stuhmer, Methods in Enzymology (1992) 207: 319-339). Oocytes are impaled with a voltage-sensing electrode and a current-sensing electrode; the voltage, or potential  
25 difference across the oocyte membrane, is clamped to a particular value using

the voltage-sensing electrode and the current, or the flow of ions across the oocyte membrane, required to maintain that voltage is measured using the current-sensing electrode. The OpusXpress system is a semi-automated two-electrode voltage clamp workstation that allows recordings to be made from 8 5 oocytes simultaneously. Oocyte impalement is automated and compound delivery is performed by computer-controlled fluid handlers from 96-well compound plates. This medium-throughput system dramatically increases the number of compounds we can examine from ~1 compound per week using a conventional single oocyte voltage clamp system to ~60 compounds per week 10 using the OpusXpress system.

[00163] Oocytes are placed in the OpusXpress system and bathed in ND-96 solution (96 mM NaCl, 2.5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES pH 7.5 with NaOH). Oocytes are impaled with voltage-sensing and current-sensing electrodes, pulled on a Model P-97 Flaming/Brown. 15 Micropipette Puller (Sutter Instrument Co.) using borosilicate glass capillaries (World Precision Instruments Inc.) and back-filled with 3M KCl, containing silver chloride wires. Electrodes exhibit resistances between 2-10 Mohm for voltage-sensing electrodes and between 0.5-2 Mohm for current-sensing electrodes. Following impalement, oocytes are voltage clamped to -60 mV 20 and experimental recordings are initiated. Data are acquired at 50 Hz and low-pass filtered at 5 Hz using a 4-pole Bessel filter.

[00164] A preferred procedure used to screen a compound for ENaC enhancement in an oocyte assay according to the invention is as follows (Figure 8). First, amiloride is applied (1 uM; Sigma). Amiloride is an inhibitor

that blocks sodium transport through ENaC channels and is used as an internal control to verify that the oocytes express functional ENaC protein (Canessa, et al, *Nature* (1994) 367: 463-467). Second, in oocytes exhibiting amiloride inhibition, evidenced by a decrease in current flowing across the 5 oocyte membrane following amiloride treatment, a compound is applied (concentration between ~0.1 uM and ~100 uM). If the compound functions as an ENaC enhancer, the current passing through ENaC channels in the oocyte membrane increases. To quantitate the effect of a compound on ENaC function, we use the following formula:  $[(A-A_0)/(B-B_0)] \times 100$ , where A is the 10 current following compound treatment,  $A_0$  is the current preceding compound treatment, B is the current following amiloride treatment, and  $B_0$  is the current preceding amiloride treatment (Figure 8). This value leads to a % enhancement factor that is used to gauge the activity of compounds in our assay. For example, if the % enhancement factor is equal to 100%, then the 15 compound increases ENaC activity 100% over basal control values (in the absence of compound). % enhancement factors are calculated individually for each of the oocytes in the OpusXpress system and then an average and standard deviation are determined for each compound (Figure 9).

[00165] Negative control experiments are performed in oocytes not injected 20 with ENaC cRNA to demonstrate that effects observed with compounds in ENaC expressing oocytes are due to currents flowing through ENaC channels and not due currents flowing through channels endogenously expressed in the oocyte membrane. (Dascal, *CRC Crit. Rev. Biochem.* (1987) 22(4): 317-387). Compounds specifically enhancing ENaC should not affect currents in

uninjected oocytes and should exhibit % enhancement factors of 0 (Figure 10).

[00166] More complex analyses are performed on compounds displaying large % enhancement factors and having no effect on oocytes not injected with ENaC cRNA. The assays include current/voltage (I/V) curves, amiloride competition experiments, and dose-response curves. For I/V curves, currents are measured in voltage steps from -100 to +60 mV, in 20 mV increments, in the presence and absence of amiloride, to verify ENaC expression as above, and in the presence and absence of compound, to investigate the magnitude of compound enhancement (Figure 11). All non-ENaC currents (defined as currents not blocked by amiloride) are subtracted and I/V curves are plotted. The slope of the I/V curve is indicative of the magnitude of current enhancement by the compound of interest. Strong enhancers exhibit I/V curves with a large positive slope. In addition, the x-intercept of the I/V curve is indicative of what type of ion is being transported in two-electrode voltage clamp experiments. For sodium ion transport, the x-intercept falls within the range of 10-40 mV, depending on the degree of sodium loading in the oocytes. In oocytes not injected with ENaC cRNA, I/V curves performed in the presence of compound should be identical and superimposable with I/V curves performed in the absence of compound (Figure 11).

[00167] Amiloride competition experiments are desirably performed to demonstrate that compound effects are ENaC dependent (Figure 12). First, amiloride is applied as above to demonstrate ENaC expression in the oocytes. Then, compound is applied to determine the % enhancement factor. Finally,

amiloride and compound are co-applied. For an enhancer to work directly on the ENaC channel, co-application of amiloride plus compound should exhibit an amiloride phenotype, meaning that currents are inhibited and not enhanced. This assay shows that when the ENaC channel is closed, due to 5 amiloride, the compound cannot have an enhancing effect; therefore, the compound must directly modulate ENaC channel function.

[00168] Dose-response curves are performed to determine the concentration at which the compound exhibits half-maximal activity (EC50) (Figure 13). The lower the EC50, the more active the compound is as an 10 ENaC enhancer. Dose-response curves are performed by sequentially applying increasing concentrations of enhancer starting from low doses (~1 nM) and progressing to high doses (~1 mM). % enhancement factors are calculated as described above and plotted as a function of compound concentration on a logarithmic scale to determine an EC50 value for the 15 compound.

[00169] A flowchart which schematically illustrates the sequence of experiments performed to examine the effect of a compound on ENaC function is depicted in Figure 14, including screening at a holding potential of -60 mV, I/V curves, amiloride competition tests, dose-response curves, and 20 testing uninjected oocytes.

### Analysis of Results

[00170] In our previous patent application (PCT WO 02/087306 A2), we utilized a high-throughput mammalian cell-based assay in HEK293T human embryonic kidney cells that indirectly measured ENaC activity by assaying

membrane voltage in ENaC-transfected cells loaded with voltage-sensitive fluorescent probes. Although this approach was high-throughput, and identified some compounds that modulated ENaC, unfortunately, it was not specific, and ~90% of identified compounds did not directly modulate ENaC

5 function but instead likely modulated the activity of other ion channels endogenously expressed in HEK293T cells. The efficacy of such high throughput assays is improved herein by the use of phenamil and similar ENaC inhibitors as described supra. In addition, the subject application also provides a more direct (specific), but lower throughput assay methodology to

10 measure ENaC sodium channel function in oocytes using the two-electrode voltage clamp technique. This system allows rapid and robust expression of ion channels (~1 million ENaC channels can be expressed in the oocyte membrane after only about 18-24 hours). Other advantages of the oocyte expression system include: oocytes are large (~1 mm in diameter) and robust

15 making them easy to handle and work with; multiple and repetitive recordings can be obtained from the same oocyte to the same or different compounds of interest; oocytes express few endogenous channels at levels sufficient to cause high background current in comparison to the current stemming from an exogenously expressed ion channel; and oocytes allow direct

20 measurement of ion channel function. Thus, in contrast to assays that indirectly measure ENaC function in HEK293T cells using voltage-sensitive probes, the oocyte expression system allows direct measurement of ENaC sodium channel current with virtually no background.

[00171] NUCLEIC ACID AND AMINO ACID SEQUENCES OF ENaC  
SUBUNITS USED IN EXAMPLES

**SEQ ID NO: 1**

Length 2010 nucleotides

DNA

Human hENaC alpha clone #3-1-1 coding sequence

atggaggggaacaagctggaggagcaggactctagccctccacagtcactccagggctcatgaaggg  
gaacaagcgtga  
ggagcaggggctgggccccgaacctgcggcgccccagcagcccacggcggaggaggaggccctgat  
cgagttccaccgcgtccctaccgagagcttcgagttctgcacaacaacaccacatccacggcgccatccg  
cctggtgtgtcccccagcacaac  
cgcatgaagacggcctctgggcagtgcgtggctctgcaccttggcatgtatgtactggcaattcggcctgct  
ttcgg  
agagtacttcagctaccccgtagccctaacaatcaacctcaactcggacaagctcgtttcccgcaactgac  
catctgca  
ccctcaatccctacaggtacccggaaattaaagaggagctggaggagctggaccgcacagagcag  
acgctttgac  
ctgtacaaaatacagctccctcaccactctcgccggctcccgacgcgtcgacactgcggggactctg  
ccgcaccc  
cttcgcgcctgagggtccgcggccctcacggggccctcgagccctgtagcgtggccctccagcttg  
cgggacaaca  
accccccagggtggacttggaaaggacttggaaagatcggcttccagctgtgcaaccagaacaaatcggactgtt  
ctaccagaca  
tactcatcagggtggatgcggtgaggagttggatccactacatcaacatcctgtcgaggctgcca  
gagactct  
gccatccctggaggaggacacgctggcaacttcatctcgccgtcaaccaggctcgcacacca  
ggcaatt  
acttcactccaccacccgatgtatggaaactgtctatactttcaatgacaagaacaactccaacctctggat  
gtctcc  
atgccttggaaatcaacaacacggctgtccctgatgcgtcgacagcagaatgtacttcatccctgtgtcca  
cagtac  
tggggcccggtaatggtcacggcaggatgaacctgccttatggatgtggcttaacttgcggct  
ggcgtgg  
agacccatcagcatgaggaaggaaaccctggacagacttggggcgattatggcactgcaccaaga  
atggcagtgt  
gttcctgttggaaaccttaccctcaaagtacacacagcagggtgtattcactcctgtccaggagagcat  
gatcaa  
ggagtgtggctgtgcctacatcttctatccgcggccccagaacgtggagttactgtgactacagaaagcaca  
gttcctgg  
ggtactgtactataagctccagggtgacttcctcagaccacctggctgtttcaccatgtccggaaagcc  
atgcagc  
gtgaccagctaccagctctgtggttactcacgtggccctcggtgacatcccaggaaatgggtcttccaga  
tgctatc  
gcgacagaacaattacaccgtcaacaacaagagaaatggagtggccaaagtcaacatcttcaagga  
gctgaactaca

aaaccaattctgagtctccctctgtcacgatggtaccctcctgtccaacctggcagccagtgaggcctgtg  
gttcggc  
tcctcggttgtctgtggtagatggctgagctcgcttgcacctgctggcatcatgttcctcatgctgctccg  
aag  
gttccgaagccgatactggtctccaggccgagggggcaggggtgctcaggaggtagcctccaccctggc  
atcctccctc  
cttcccactctgccccccccatgtctgtcctgtcccagccaggccctgctccctctccagcctgacag  
ccccct  
ccccctgcctatgccaccctggccccccatctccagggggctgcaggggccagttccaccctgt  
cctctggg  
ggggccctga

**SEQ ID NO: 2**

Length 1923 nucleotides

DNA

Human hENaC beta clone #5 coding sequence

atgcacgtgaagaagtacctGctgaaggccctgcacggctgcagaaggccccggctacacgtacaa  
ggagctgctgg  
gtggtaactgcgacaacaccaacacccacggcccaagcgcatcatctgtgagggggccaaagaagaaag  
ccatgtggttcc  
tgctaccctgcttcgccccctcgctgtggcagtggggcatcttcatcaggacctacttgagctggag  
gtcagc  
gtccctctccgtaggctcaagaccatggactccccgccgtcaccatctgcaatgtctggccctcaagta  
ttccaa  
aatcaagcattgtgaaggacacctggatgagctgtatggaaagctgtcctggagagaatcctggctctgagct  
aagccatg  
ccaatgccaccaggaacctgaactctccatctggaaaccacacacccctggcatttgcatacgaaacggaaac  
ccccaccac  
cccatggtcctgatctttggagacaaccacaatggcttaacaaggcagctcagcatcagaaaagatctgt  
aatgcca  
cgggtcaaaatggccatgagactatgtacccctcaacaggaccaggactgtacccctggaaacttcaccagg  
ctaccagg  
cattgacagagtggtacatcctgcagggccaccaacatcttgcacagggccacagcaggagcttaga  
gatgagctac  
cccgccgagcagatgatccctggcctgcattcggagctgagccctgcaactaccggaaacttcacgtccat  
cttctaccc  
tcactatggcaactgttacatctcaactggggcatgacagagaaggactccctggccaaaccctggaaac  
tgaattcg  
gcctgaagttgatccctggacataggccaggaagactacgtcccttcgtccacggccgggtcagg  
ctgatgtt  
cacgagcagaggcataccctcatcagagatgagggcatctacGccatgtcggggacagagacgtcc  
atcggggact  
cgtggacaagcttcagcgcatggggagccctacagccgtgcaccgtgaatggtctgaggccccgtcc  
aaaactct  
acagtgactacaacacgacactccatccaggccgtctcgctcgtccatgcaacaaccggacttccagactgg  
ctgcaac  
tgtggccactacgttacccactGcccggtggggagaaatactgcaacaaccggacttccagactgg  
cccattgcta

ctcagatctacagatgagcgtggcgagagagagacctgcattggcatgtgcaaggagtccctgcaatgac  
acccagtaca  
agatgaccatctccatggctgactggcctctgaggcctccgaggactggatttccacgtctgtctcaggag  
cgggac  
caaagcaccaatatcaccctgagcaggaagggattgtcaagctcaacatctActtccaagaatttaacta  
tcgcaccat  
tgaagaatcagcagccaataacatcgctggctctcgaaatctgggtggccagttggctctggatgggg  
ggctctg  
tgctgtgcctcatcgagttggggagatcatcatcgacttgcgtggatcaccatcatcaagctggccttgg  
ccaag  
agcctacggcagcggcgagcccaagccagCtacgctggcccaccgcccacccgtggccagctggtgg  
aggccacaccaacttggctccagcctgacacggccccccgagcccaacactggccctacccca  
gtgagcaggccctgcccattccag  
gcaccccccaccaactatgactccctgcgtctgcagccgctggacgtcatcgagtcacagtgagggt  
gatgccatc  
taa

**SEQ ID NO: 3**

Length 1950 nucleotides

DNA

Human hENaC gamma clone #3 coding sequence

atggcacccggagagaagatcaaaggccaaatcaagaagaatctgcccgtacggggccctcaggcgc  
cgaccattaaaga  
gctgatgcgggtggactgcctcaacaccaacacccatggctgcgcgcacgtgggtgtccgcggccgtct  
gcgcgc  
tcctctggatcgggtcacactgactgcgtggccctcatcctctggcagtgcgcctcctcgtcttccttat  
act  
gtctcagttccatcaaagtccacttccggaagctggatttcctgcagtcaccatctgcaacatcaaccccta  
caagta  
cagcacccgttcgcaccccttagctgacttggaaacaggagaccagagaggccctgaagtccctgtatggctt  
tccagagt  
cccgaaagcgccgagaggcggagtccgtggactccgtctcagaggaaagcagcctagattctccacc  
ggattccgctg  
ctgatcttgcaggatgagaaggcaaggccaggacttccacagggAggaagcggaaagtccgc  
ggtagcatcat  
tcacaaggctcaaatgtcatgcacatcgacttccaaagcaagtgggtggattccaaactgtgctcaaatgacac  
ctccgact  
gtgccacccatccctcagctggaaatcaatgcattcaggagtggataagctacactacatgaacatca  
tggcacag  
gtgcctctggagaagaaaatcaacatgagctattctgctgaggagactgtggtgcacccgttcttgc  
gtcctg  
tgcgcaggaaattcacgccttCcaccacccgatgcattggaaatgcataacttcaacaacagagaaaat  
gagacca  
ttctcagcacccatggggcagcgaatatggctgcaggacttgcatttgcataaaacgaagagaaat  
aacccattc  
ctcggtccactggagctaaaggatcatccatccgcaggatgaggatccctgcgtcaagatgtggaa  
cagagat  
tgagacagcaatggtcacccataggaatgcacccgtacagagtcctcaagctgagtcacgcctacagtc  
agtgcacgg

aggacgggagtgacgtgccaatcaggaacatctacaacgctgcctactcgctccagatctgccttcattcat  
gcttccag  
acaaagatggtagaaatgtgggtgcccagtagccgccttacccctgcagccaactactgca  
actaccagca  
gcaccccaactggatgtattttactaccaactgcatcgagcccttgcaggaagagctggctgcagtc  
gtgtca  
aggaagcctgcagcttaaagagtggacactaaccacaagcctggcacaatggccatctgtggttcggag  
aagtggtg  
ctgccttctcacttggaccaaggccggcaagtaaaaaagctcaacaagacagacttgGccaaa  
ctcttgatatt  
ctacaaagacctaaccagagatccatcatggagagccagccaacagtattgagatgctctgtccaact  
tcggtgcc  
agctgggcgtggatgagctgtctgtgtcgcatcgagatcatcgaggcttgcattgacttctctcta  
tc  
attgcccgcgcaggcaggaaagccaaggagtggggcctggaaacaggctccccatgtccaga  
agctccccgtag  
cccacaggccaggacaatccagccctggatagacgatgacccacttcaactctgtttgcacct  
gcctccaG  
cccttaggaacccaagtgcggcacaccgcccccaaatacaatacccttgcgtggagagggctttcc  
aaccagctc  
acagatacccagatgctAgatgagctctga

**SEQ ID NO: 4**

Length 669 amino acids

PRT

Human hENaC alpha clone #3-1-1 amino acid sequence  
MEGNKLEEQDSSPPQSTPGLMKGNKREEQGLGPEPAAPQQPTAEEEALIE  
FHRSYRELFEFFCNNTTIHGAIRLVCSQHNRMKTAFWAVLWLCTFGMMYW  
QFGLLFGEYFSYPVSLNINLNSDKLVFPAVTICTLPYRPEIKEELEELDRIT  
EQTLFDLYKYSSFTTLVAGSRSRRDLRGTLPHPLQRLRVPPPHGARRARS  
VASSLRDNNPQVDWKDWKIGFQLCNQNKSDFYQTYSSGVDAREWYRF  
HYINILSRLPETLPSLEEDTLGNFIFACRFNQVSCNQANYSHFHHPMYGN  
TFNDKNNSNLWMSSMPGINNGLSLMLRAEQNDFIPLLSTVTGARVMVHGQD  
EPAFMDDGGFNLRPGVETSI  
SMRKETLDRLGGDYGDCTKNGSDVPVENLY  
PSKYTQQVCIHSCFQESMIKECGCAYIFYPRPQNVEYCDYRKHSSWG  
YCYKLQVDFSSDHLCFTKCRKPCSVTSYQLSAGYSRWPSVTSQEWF  
QMLSRQNNYTVNNKRNGVAKVN  
IFFKELNYKTNSESPSVTMTLLSNLGSQWSLWF  
GSSVLSV  
VEMAELVFDLLVIMFLMLRRFRSRYWSPGRGRRGAQE  
VASTLA  
SSPPSHFCPHMSLSSQPGPAPSPALTAPPAYATLGPRPSPGG  
SAGASS  
STCPLGGP

**SEQ ID NO: 5**

Length 640 amino acids

PRT

Human hENaC beta clone #5 amino acid sequence

MHVKKYLLKGLHRLQKGPGYTYKELLVWYCDNTNTHGPKRIICEGPKKKAM  
WFLLTLLFAALVCWQWGFIRTYLSWEVSVSLSVGFKTMDFPAVTICNASPF  
KYSKIKHLLKDLDELMEAVLERILAPELSHANATRNLNFSIWNHTPLVLIDERN  
PHHPMVLDLFGDNHNGLTSSASEKICNAHGCKMAMRLCSLNRTQCTFRN  
FTSATQALTEWYILQATNIFAQVPQQELVEMSYPGEQMIACLFGAEPCNYR  
NFTSIFYPHYGN CYIFNWGMTEKALPSANPGTEFGLKLILDIGQEDYVPFLAS  
TAGVRLMLHEQRSYFIRDEGIYAMSGTETSIGVLVDKLQRMGEPYSPCTVN  
GSEVPVQNFYSDYNTTYSIQACLRSCFQDHMIRNCNCGHYLYPLPRGEKYC  
NNRDFPDWAHCYSDLQMSVAQRETCIGMCKESCNDTQYKMTISMADWPS  
EASEDWIFHVLSQERDQSTNITLSRKGIVKLNIFYFQE NYRTIEESAANNIVWL  
LSNLGGQFGFWMGGSVLCLIEFGEIIIDFVWITIILVALAKSLRQRRAQASYA  
GPPPTVAELVEAHTNFGFQPD TAPRSPNTGPYPSEQALPIPGT PPPNYDSL  
RLQPLDVIESDSEGDAI

**SEQ ID NO: 6**

Length 650 amino acids

PRT

Human hENaC gamma clone #3 amino acid sequence

MAPGEKIKAKIKKNLPVTGPQAPTIKELMRWYCLNTNTHGCRRIIVSRGRLR  
RLLWIGFTLTAVALILWQCALLVFSFYTVSVSIKVHFRKLDPAVTICNINPYKY  
STVRHLLADLEQETREALKSLYGFPE SRKRREAESWNSVSEGKQPRF SHRI  
PLLI FDQDEKGKARDFFTGRKRKVGGSIHKASNVMHIESKQVVGFLQLCSND  
TSDCATYTFSSGINAIQE WYKLHYMNIMAQVPLEKKINMSYSAEELLVTCFFD  
GVSCDARNFTLFHHPMHGNCYTFNNRENETILSTSMGGSEYGLQVILYINEE  
EYNPFLVSSTGAKVIIHRQDEYPFVEDVGTEIETAMVTSIGMHLTESFKLSEP  
YSQCTEDGSDVPIRNIYNAAYSLQICLHSCFQTKMVEKCGCAQYSQPLPPAA  
NYCNYQQHPNW MYCYYQLHRAFVQEELGCQSVC KEACSFKEWTLTSLA  
QWPSVVSEKWL PVL TWDQGRQVNKKLNKTDLA KLLIFYKDLNQR SIMESP  
ANSIEMLLSNFGGQLGLWMSCSVCVIEIIEVFFIDFFSIIARRQWQKAKEWW

AWKQAPPCPEAPRSPQGQDNPALDDDLPTFNSALHLPPALGTQVPGTPP  
PKYNTLRLERAFSNQLTDTQMLDEL

**SEQ ID NO: 7**

Length 1917 nucleotides

DNA

gi|1066456|gb|U38254.1|HSU38254 Human amiloride sensitive sodium channel delta subunit ( $\Delta$ NaCh) mRNA, complete coding sequence

ATGGCTGAGCACCGAACGATGGACGGGAGAATGGAAGCAGCCACACGG  
GGGGGCTCTCACCTCCAGGCTGCAGCCCAGACGCCCGGG  
GCCACCATCAGCACCAACCACCACCCAAAGGAGGGCACCAGGAGGG  
GCTGGTGGAGCTGCCGCCTCGTCCGGGAGCTGCTCACCTTCTGC  
ACCAATGCCACCATCCACGGGCCATCCGCCTGGTCTGCTCCCGGG  
AACCGCCTCAAGACGACGTCCTGGGGCTGCTGTCCCTGGAGCCCTG  
GTCGCGCTCTGCTGGCAGCTGGGGCTCCTCTTGAGCGTCACTGGCAC  
CGCCCGGTCCATGCCGTCTGTGCACTCGGAGCGCAAGCTGCTC  
CCGCTGGTCACCCTGTGTGACGGGAACCCACGTCGGCCAGTCCGGTC  
CTCCGCCATCTGGAGCTGCTGGACGAGTTGCCAGGGAGAACATTGACT  
CCCTGTACAACGTCAACCTCAGCAAAGGCAGAGCCGCCCTCTCCGCCAC  
TGTCCCCGCCACGAGCCCCCTTCCACCTGGACCGGGAGATCCGTCT  
GCAGAGGCTGAGCCACTCGGGCAGCCGGTCAGAGTGGGTTAGACT  
GTGCAACAGCACGGCGGACTGCTTTACCGAGGCTACACGTCAGG  
CGTGGCGGCTGTCCAGGACTGGTACCACTTCCACTATGGATATCCTG  
GCCCTGCTGCCCGGGCATGGGAGGACAGCCACGGAGGCCAGGACGG  
CCACTTCGTCCCTCCTGCAGTTACGATGGCCTGGACTGCCAGGCCGA  
CAGTTCCGGACCTTCCACCAACCCACCTACGGCAGCTGCTACACGGTCG  
ATGGCGTCTGGACAGCTCAGCGCCCCGGCATACCCACGGAGTCGGCC  
TGGCCTCAGGGTTGAGCAGCAGCAGCCTCACCTCCCTTGCTGTCCACGCT  
GGCCGGCATCAGGGTCATGGTCACGGCGTAACCACACGCCCTTCCT  
GGGGCACCACAGCTTCAGCGTCCGGCCAGGGACGGAGGCCACCATCAG  
CATCCGAGAGGACGAGGTGCACCGGCTGGGAGGCCCTACGGCCACTG  
CACCGCCGGCGGGGAAGGCGTGGAGGTGGAGCTGCTACACAACACCTC  
CTACACCAGGCAGGCCTGCCTGGTCTGCTTCCAGCAGCTGATGGTG  
GAGACCTGCTCCTGTGGCTACTACCTCCACCCCTTGCCGGCGGGGCT

GAGTACTGCAGCTCTGCCCGCACCCCTGCCTGGGGACACTGCTTCTACC  
GCCTCTACCAGGACCTGGAGACCCACCGGCTCCCTGTACCTCCGCT  
GCCCGAGGCCCTGCAGGGAGTCTGCATTCAAGCTCTCCACTGGGACCT  
CCAGGTGGCCTTCCGCCAAGTCAGCTGGATGGACTCTGGCCACGCTAG  
5 GTGAACAGGGGCTGCCGCATCAGAGCCACAGACAGAGGAGCAGCCTGG  
CCAAAATCAACATCGTCTACCAGGAGCTCAACTACCGCTCAGTGGAGGA  
GGCGCCCGTGTACTCGGTGCCGCAGCTGCTCTCCGCCATGGCAGCCT  
CTACAGCCTGTGGTTGGGCCTCCGTCTCCCTGGAGCTCCTG  
GAGCTGCTGCTCGATGCTTCTGCCCTCACCCCTGGTGCTAGGCAGGCCGC  
10 CGGCTCCGCAGGGCGTGGTTCTCCCTGGCCCAGAGCCAGCCCTGCCCTCA  
GGGGCGTCCAGCTCAAGCCAGAGGCCAGTCAGATGCCCGCCTGCAG  
GCGGCACGTCAGATGACCCGGAGCCCAGCGGGCCTCATCTCCCACGGG  
TGATGCTTCCAGGGTTCTGGCGGGAGTCTCAGCCGAAGAGAGAGCTGGG  
CTGGCCCCAGCCCTTGAGACTCTGGACACCTGA

15 **SEQ ID NO: 8**

Length 638 nucleotides

PRT

gi|1710872|sp|P51172|SCAD\_HUMAN Amiloride-sensitive sodium channel

delta-subunit amino acid sequence (Epithelial Na<sup>+</sup> channel delta subunit)

20 (Delta ENaC) (Nonvoltage-gated sodium channel 1 delta subunit) (SCNED)  
(Delta NaCh)

MAEHRSMMDGRMEAATRGGSHLQAAAQTPPRPGPPSAPPPPKEGHQEGL  
VELPASFRELLTFCTNATIHAIRLVCSRGNRLKTTSWGLLSLGALVALCW  
QLGLLFERHWHRPVLMAVSVHSERKLLPLVTLCDGNPRRPSPVLRHLELLD

25 EFARENIDSLYNVNLSKGRAALSATVPRHEPPFHLDREIRLQRLSHSGSRVR  
VGFRLCNSTGGDCFYRGYTSVAAVQDWYHFHYVDILALLPAAWEDSHGS  
QDGHFVLSCSYDGLDCQARQFRTHHPTYGSCYTVDGWVTAQRPGITHGV  
GLVLRVEQQPHLPLLSTLAGIRVMVHGRNHTPFLGHHSFSVRPGTEATISIRE  
DEVHRLGSPYGHCTAGGEGVEVELLHTNSYTRQACLVSCFQQLMVETCSC

30 GYYLHPLPAGAEYCSSLARHPAWGHCFYRLYQDLETHRLPCTSRCPRPCRE  
SAFKLSTGTSRWPSAKSAGWTLATLGEQGLPHQSHRQRSSLAKINIVYQEL  
NYRSVEEAPVYSPVQQLSAMGSLYSLWFGASVLSLLELLLDASALTLVLG  
GRRLRRAWFSWPRASPASGASSIKPEASQMPPPAGGTSDDPEPSGPHLPR  
VMLPGVLAGVSAEESWAGPQPLETLDT

[00172] While the invention has been described by way of examples and preferred embodiments, it is understood that the words which have been used herein are words of description, rather than words of limitation. Changes may 5 be made, within the purview of the appended claims, without departing from the scope and spirit of the invention in its broader aspects. Although the invention has been described herein with reference to particular means, materials, and embodiments, it is understood that the invention is not limited to the particulars disclosed. The invention extends to all equivalent structures, 10 means, and uses which are within the scope of the appended claims.